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Improvement of Salt Tolerance in Tomato Plant (*Lycopersicon esculentum*) by Transformation with Ectoine Biosynthetic Genes

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ABSTRACT

Ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidinecarboxylic acid) biosynthetic genes (*ect ABC)* derived from the halophilic bacterium *Halomonas elongata* were introduced into tomato (*Lycopersicon esculentum*) using an *Agrobacterium*-mediated gene-delivery system. Stable integration of the ectoine genes into the regenerated plant genomes was confirmed by PCR and Southern blot analyses. Expression of these genes was detected in the transgenic tomato plants by Northern blot analysis. The transgenic plants exhibited the normal growth characteristics of the non-transgenic plants. The concentration of ectoine increased with increasing salinity, and the increase was higher in the roots than in the leaves. The present data indicates that the turgor values of the ectoine transgenic tomato lines increased with increasing salt concentration. The data suggests that the accumulation of ectoine in transgenic tomato plants contributed to the maintenance of osmotic potential of the cells.

Keywords: *Agrobacterium*, gene expression, NMR analysis, osmotic potential

Abbreviations: AS, Acetosyringone; **MS**, Murashige and Skoog medium; **NMR**, nuclear magnetic resonance; **OA**, osmotic adjustment; Ψ_w, water potential of the cell

INTRODUCTION

Agricultural productivity is severely affected by soil salinity, and the damaging effects of salt accumulation in agricultural soils have influenced ancient and modern civilizations. The detrimental effects of salt on plants are a consequence of both water deficits resulting in osmotic stress and the effects of excess sodium ions on key biochemical processes. In response to salinity stress, plants accumulate low-molecular weight osmolytes such as sugar alcohols (e.g. glycerol, sorbitol and mannitol), and specific amino acids (proline and the quaternary ammonium compound glycine betaine). Transgenic plants harboring genes for the biosynthesis of mannitol, proline, ononitol, trehalose, betaine and fructan have shown a significant improvement in water stress tolerance (Romero *et al*. 1997; Sheveleva *et al*. 1997; Moghaieb *et al*. 2000).

Ectoine is one of the most common compatible solutes in halophilic bacteria (Csonka and Epstein 1996). The role and activity of ectoine are of special interest because it can be synthesized *de novo* in bacterial cells. Its synthesis in a number of *Streptomyces* strains in response to increased salinity and elevated temperature has been described (Malin and Lapidot 1996). Much information has been accumulated on ectoine activity in living cells. It has been shown that exogenous ectoine can reverse the growth inhibition caused by osmotic stress in *Escherichia coli* (Jebbar *et al.* 1992), *Corynebacterium glutamicum* (Farwick *et al.* 1995), and the soil bacterium *Rhizobium meliloti* (Talibart *et al.* 1994).

Because an important feature of osmoprotectants is that their beneficial effects are generally not species-specific, alien osmoprotectants can be introduced into plants to protect their new host (Rontein *et al.* 2002). Transgenic plants harboring genes for the biosynthesis of mannitol (Tarczynski *et al.* 1993), proline (Kishor *et al.* 1995), ononitol (Sheveleva *et al.* 1997), trehalose (Romero *et al.* 1997), betaine (Hayashi *et al.* 1997; Sakamoto *et al.* 1998; Moghaieb *et al.* 2000), fructan (Pilon-Smits *et al.* 1995), and ectoine (Moghaieb *et al.* 2006) have proven more tolerant to salt stress than their wild-type counterparts.

The ectoine biosynthetic pathway in the halophilic bacterium *Halomonas elongate* consists of three steps. The first step, conversion of aspartate β-semialdehyde (ASA) to L-2, 4-diaminobutyric acid (DABA), is catalyzed by 2,4-diaminobutyrate aminotransferase (DAT). The second step, which is the acetylation of DABA to N^{γ}-acetyl L-2,4-diaminobutyric acid (ADABA), is promoted by DABA acetyltransferase (DAA). In the last step, ectoine synthase (ES) catalyzes the cyclic condensation of ADABA to ectoine (Ono *et al*. 1999).

The objectives of the present study were to transform tomato plants with ectoine biosynthetic genes and to evaluate the improvement in salt tolerance in terms of biomass production and osmotic potential of the plant cells.

MATERIALS AND METHODS

Co-cultivation

Tomato seeds (*Lycopersicon esculentum* L cv. 'Momotaro' and cv. 'Saturn') were germinated on Murashige and Skoog (MS) medium. Hypocotyl explants were excised from 6-day-old seedlings and used for ectoine transformation. *Agrobacterium tumefaciens* strain LBA4404 cells harboring the binary Ti vector pBI101 Hm *ect. ABC* (Nakayama *et al.* 2000; **Fig. 1**) were grown overnight in 30 ml of LB medium containing 50 μg/ml kanamycin sulfate (Sigma Aldrich, Japan) and then collected by centrifugation at 1120 x *g* for 5 min. The pellet was re-suspended in MS medium containing 100 μM acetosyringone (AS). The hypocotyl explants prepared from 6-day-old seedlings were immersed in the bacterial suspension for 5 min. Then, the explants were blotted with sterilized filter paper and placed on a co-cultivation medium consisting of MS medium (Murashige and Skoog 1962) with 100 μM AS and supplemented

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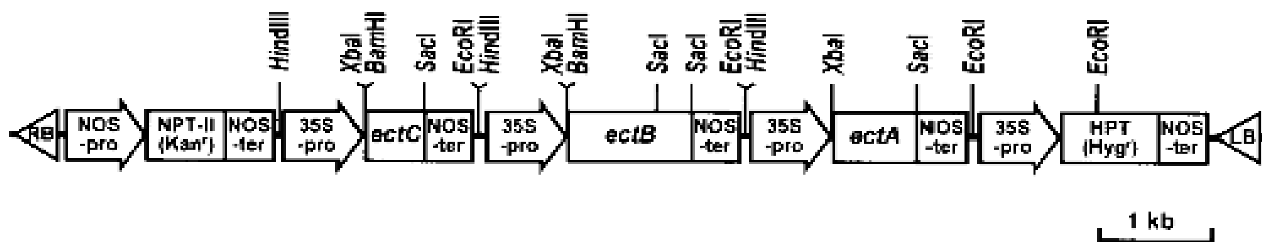


Fig. 1 Schematic representation of the pBI-101 Hm *ect. ABC* plasmid.

with 2 mg L^{-1} *trans*-zeatin. Three weeks later, adventitious shoots emerged from the cut ends of the explants. The regenerated shoots were able to produce roots on the same medium. The cultures were incubated at 25°C under a 16/8 h light/dark photoperiodic regime (70 μ mol m^{-2} s^{-1}) and sub-cultured weekly on corresponding freshly prepared medium. T_0 plantlets that had regenerated *in vitro* were transferred to pots and grown to maturity in a greenhouse. T_1 seeds obtained by self-pollination of T_0 plants were surface-sterilized and germinated on MS medium containing 50 mg L^{-1} kanamycin. The germinated seedlings were transferred to a greenhouse and used for Southern and Northern blot analyses.

PCR analysis

Genomic DNA was isolated from both transformed and non-transformed plants according to a method previously described by Rogers and Bendich (1985) and analyzed by PCR using specific primer for the *Ect C* gene. The forward and reverse primers for the *Ect C* gene were: 5'-CACTGGAGG ATCCACATGATCGTTC-3' and 5'-CAGAATAGAGCTCCGGGTTAC AGCG-3' respectively. The reaction mixture (20 μl) contained 10 ng DNA, 200 mM dNTPs, 1 mM of each primer, 0.5 units of Red Hot Taq polymerrase (ABgene Housse, UK) and 10-X Taq polymerase buffer (ABgene Housse, UK). The following profile was used for these reactions: 94°C/1 min, followed by 30 cycles of 98°C/20 s, 68°C/ 1 min 30 s, and a final extension at 72°C/10 min. PCR products were separated by (2%) agarose gel electrophoresis and visualized with ethidium bromide.

Southern blot analysis

Total genomic DNA was isolated from both transformed and non-transformed plants according to a method previously described by Rogers and Bendich (1985). Using standard protocols, 10 μg DNA was digested with restriction enzyme (*Bgl*II), and then the fragments were separated on 1% agarose gels, and blotted onto nylon membranes (Sambrook *et al.* 1989). Labeling of the probes, hybridization, and detection were carried out using an RPN 3540 Gene Image kit according to manufacturer's instructions (Amersham, Buckinghamshire, UK).

Northern blot analysis

Total RNA was isolated from leaf samples from both the transgenic and control plants using the method of Chrigwin *et al.* (1979), and Northern hybridization was carried out using an RPN 3450 Gene Image kit according to manufacturer's instructions (Amersham, Buckinghamshire, UK).

Plant growth and salt treatments

For the salt tolerance experiments, non-transformed tomato seeds from the cultivar 'Momotaro' (MWT) and the cultivar 'Saturn' (SWT) and three independent transgenic lines (T_2) (MT, S1 and S2) derived from the two cultivars were used. Seedlings were transferred into 3-l plastic pots containing a mixture of granite regosol, peat moss, and perlite (2:1:1, v/v/v). Seedlings were irrigated daily with 400 ml of 10% Hoagland's solution (Hoagland and Arnon 1950), and the soil water tension was maintained at ≤ 60 kP_a. At 30 days after planting, the plants were subjected to salt stress by the addition of 100, 200 or 300 mM NaCl to the daily supply of Hoagland's solution for one week. The temperature in the greenhouse was 28°C, and the photosynthetically active radiation (PAR) was 2743 μmol m^{-2} s^{-1}. There were five replicates per treatment.

Measurements of plant dry weight

Plants were harvested one week after initiation of the salt treatment. The harvested plants were separated into leaves, stems and roots, which were individually dried at 80°C in an air-forced draught oven for more than three days, and then weighed.

Na^+, K^+ and ectoine analysis

The freeze-dried samples were ground into a fine powder. Samples were extracted with 10 ml of 1 N HCl for 24 h at room temperature. The Na^+ and K^+ concentrations of the extracts were determined using a flame photometer (Eiko Instruments, Tokyo).

Methanol-extracted ectoine was purified by ion-exchange chromatography according to Yang *et al.* (1995) and analyzed by ^{1}H nuclear magnetic resonance (NMR) spectroscopy using a JEOL-GSX 500 NMR instrument. The amount of ectoine present was calculated by comparing the peak area of ectoine with that of the internal standard (formate).

Determination of leaf water relations

Leaf water potential (ψ_w) was measured by using a pressure chamber (Daiki-Rika Instruments, Tokyo, Japan) one week after salt treatment started. After measurement of ψ_w, the leaf samples were frozen in liquid nitrogen and stored at -20°C. Leaf tissues were thawed and centrifuged at 1,200 x *g* for 25 min at 4°C to extract the cell sap. The osmotic potential (ψ_s) of the cell sap was measured using a vapor pressure osmometer (model 5,500; Wescor, Logan, UT, USA). Turgor potential was calculated by subtracting ψ_s from ψ_w. Osmotic adjustment (OA) was calculated as the difference in ψ_s between salinized and control plants. The number of replication was five per salt treatment.

Statistical analysis

Statistical analysis was performed using the Analyze-it software (Analyze-it, Leeds, UK) accordance to the method of Maxwell and Delany (1989).

RESULTS AND DISCUSSION

Cultivated tomato is moderately sensitive to salinity (Cuartero *et al.* 1992), relatively high salt tolerance was found in some wild *Lycopersicon* species namely *L. cheesmanii*, *L. pennellii* and *L. peruvianum* (Saranga *et al.* 1992). To improve the salt tolerance in cultivated tomato, in the present study the hypocotyl explants isolated from two tomato cultivars (Momotaro and Saturn) were transformed with the ectoine biosynthetic genes (*ect ABC*) using an *A. tumefaciens*-mediated gene-delivery system. In order to confirm the presence of the ectoine biosynthetic genes in the putative transgenic plant genomes, all the obtained plants were subjected to PCR analysis with the primers specific for the *Ect C* gene. The PCR analysis indicated that clear band corresponding to the relevant sequence of the *Ect C* gene (432 bp) was detected only in the ectoine transgenic plants (**Fig. 2A**). The presence of transgenes in the regenerated plants was also confirmed by Southern blot analysis, in which the transgenic plants yielded 1-3 bands that

hybridized specifically with the *Ect B* gene (**Fig. 2B**).

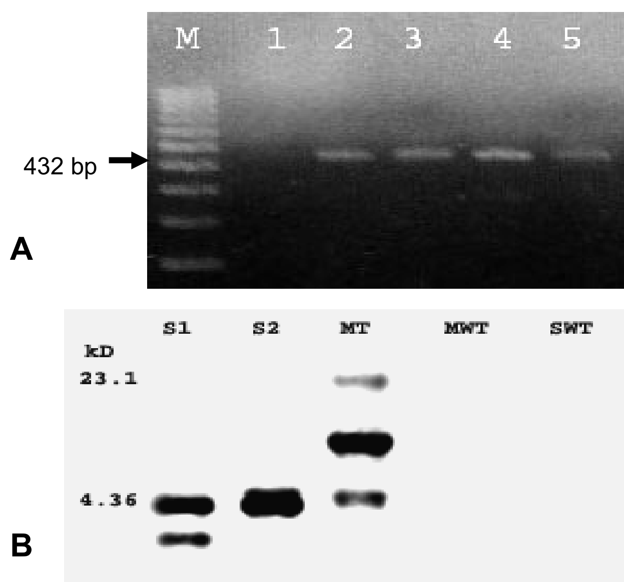


Fig. 2 PCR and Southern blot analysis confirming the integration of the ectoine biosynthetic genes in transgenic plant genome. (**A**) PCR analysis of transgenic plants using *Ect C* gene-specific primers, M, 100 bp DNA marker, Lane: 1 non-transgenic plant and Lanes 2-5 are transgenic plants. (**B**) Southern blot hybridization of DNA prepared from transgenic tomato plants transformed with the ectoine biosynthetic genes. DNA (10 µg) was cut by restriction enzymes (*Bgl* II), separated on 1% agarose, transferred to membrane and hybridized to labeled probe (*ect. B*). WT: (wild-type) and, T (transgenic) 'Momotaro' (MT), and 'Saturn' (S1, S2).

Although expression of multiple CaMV35S promoters tends to be silenced in transgenic plant cells, our data shows that at least four CaMV35S promoters could be transcribed at the same time. Furthermore, the fact that ectoine was detected in ectoine transgenic plants (and only the transgenic plants) indicates that mRNAs derived from each of the *ect* genes were successfully translated to produce the enzymes of the ectoine synthetic pathway (**Fig. 3**).

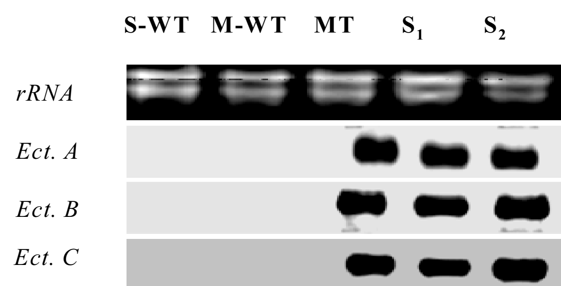
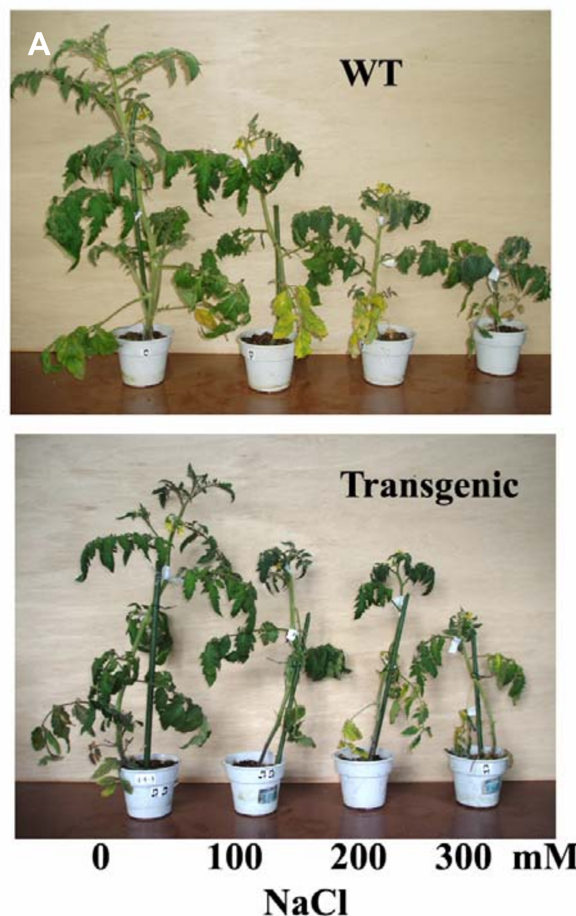


Fig. 3 Northern blot analysis, total RNA isolated from leaves both of the transgenic and non-transgenic tomato plants. Equal amounts of RNA (30 µg) were blotted to nylon membrane and hybridized with ectoine biosynthesis gene (*ect a, b, c*) probes. Ribosomal RNA (rRNA) visualized by staining with ethidium bromide, is shown as a control. Northern blotting analysis was conducted for leaves showing *Ect.A*, *Ect.B* and *Ect.C* gene expression. Lanes marked with MWT and SWT are wild-types of 'Momotaro' and 'Saturn', respectively. Lanes marked MT, S_1 and S_2 are transgenic lines.

The ectoine transgenic tomato lines (T_2) as well as the non-transgenic plants were subjected to gradual increases in NaCl concentration (0, 100, 200 and 300 mM) for one week. The growth of the transgenic lines improved under salt stress compared with the non-transgenic lines (**Fig. 4A-C**). The whole-plant dry weight decreased with increasing NaCl concentration in the culture medium, and the reduction was more marked in the non-transgenic than in



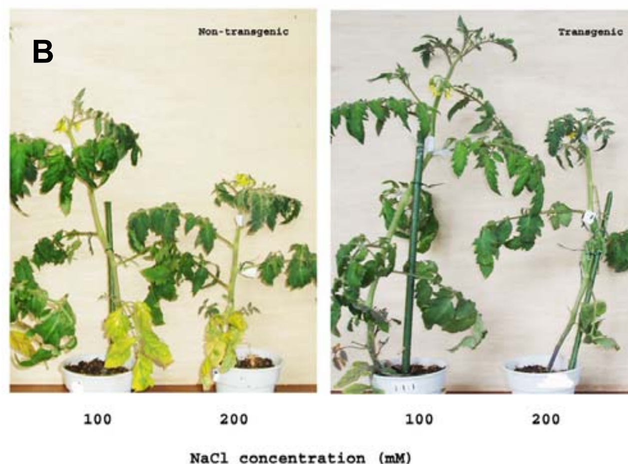


Fig. 4 Growth of tomato plants under salt stress. (**A, B**) enhancement of transgenic plant growth compared with the non-transgenic plants (WT) under salt stress; (**C**) growth of the ectoine transgenic tomato plants under 200 mM NaCl.

Table 1 Effect of salinity on plant dry weight in ectoine transgenic (T2 of line MT, S1 and S2) and wild-type (MWT and SWT) tomato plants.

Genotype	NaCl (mM)	Leaves	Stems	Roots	Whole plant	Whole plant relative DW (%)
MWT	0	7.2 ± 0.1	4.2 ± 0.2	1.7 ± 0.01	13.1	100
	100	3.1 ± 0.3	2.8 ± 0.1	0.9 ± 0.02	6.8	51.9
	200	1.9 ± 0.4	2.4 ± 0.3	0.5 ± 0.03	4.8	36.6
	300	1.3 ± 0.1	1.2 ± 0.2	0.5 ± 0.02	3	22.9
MT	0	13.2 ± 0.5	4.5 ± 0.1	2.4 ± 0.1	21	100
	100	6.4 ± 0.02	4.4 ± 0.09	1.3 ± 0.02	12.1	57.6
	200	5.1 ± 0.1	4.1 ± 0.1	0.7 ± 0.01	9.9	47.1
	300	4.2 ± 0.1	2.1 ± 0.1	0.3 ± 0.02	6.6	31.9
SWT	0	2.9 ± 0.2	1.6 ± 0.1	1.1 ± 0.05	5.6	100
	100	1.6 ± 0.1	1.2 ± 0.3	0.7 ± 0.04	3.5	62.5
	200	1.2 ± 0.2	0.9 ± 0.1	0.5 ± 0.01	2.6	44.6
	300	1.5 ± 0.2	0.8 ± 0.2	0.4 ± 0.01	2.2	48.2
S1	0	6.2 ± 0.1	2.5 ± 0.2	1.8 ± 0.02	10.5	100
	100	4.9 ± 0.1	2.8 ± 0.2	0.8 ± 0.02	8.5	80.9
	200	4.5 ± 0.3	2.0 ± 0.1	0.6 ± 0.03	7.1	67.6
	300	3.4 ± 0.1	1.6 ± 0.3	0.9 ± 0.01	5.9	56.2
S2	0	3.1 ± 0.3	1.3 ± 0.1	1.2 ± 0.06	5.6	100
	100	2.8 ± 0.1	1.3 ± 0.1	0.7 ± 0.03	4.8	85.7
	200	2.3 ± 0.2	1.1 ± 0.2	0.7 ± 0.03	4.1	73.2
	300	1.8 ± 0.1	1.2 ± 0.1	0.5 ± 0.04	3.5	62.5

Values are means of five replicates ± standard deviation.

Table 2 Effect of salinity on water relations in leaves of ectoine transgenic tomato lines (MT, S1 and S2) and wild-type (MWT and SWT) tomato plants.

Genotype	NaCl mM	Ψ_w (MPa)	Ψ_s (MPa)	Ψ_p (MPa)	OA
MWT	0	-0.785	-0.907	0.122	
	100	-1.275	-1.691	0.416	0.294
	200	-1.397	-2.011	0.613	0.491
	300	-1.471	-2.062	0.591	0.469
MT	0	-0.662	-0.873	0.212	
	100	-1.005	-1.661	0.656	0.444
	200	-1.054	-2.217	1.162	0.950
	300	-1.324	-2.607	1.283	1.071
SWT	0	-0.637	-0.790	0.153	
	100	-0.809	-1.462	0.653	0.500
	200	-1.250	-1.837	0.587	0.434
	300	-1.079	-2.005	0.926	0.773
S1	0	-0.711	-0.855	0.144	
	100	-0.809	-1.710	0.901	0.757
	200	-0.956	-2.056	1.099	0.955
	300	-0.539	-2.334	1.795	1.651
S2	0	-0.588	-0.859	0.270	
	100	-0.785	-1.429	0.644	0.374
	200	-0.932	-1.954	1.022	0.752
	300	-0.686	-2.399	1.712	1.442

Table 3 Effect of salinity on concentration (µmol g^{-1} FW) of Na^+, K^+ and ectoine in transgenic tomato lines (MT, S1 and S2) and wild-type (MWT and SWT).

Genotype	NaCl (mM)	Na^+ Leaf	K^+ Leaf	Ectoine (µmol g^{-1}FW) Roots	Leaf
MWT	0	0.5 ± 0.1	12.0 ± 0.3	0.0 ± 0.0	0.0 ± 0.0
	100	12.5 ± 0.8	12.5 ± 0.2	0.0 ± 0.0	0.0 ± 0.0
	200	17.8 ± 0.7	12.5 ± 0.1	0.0 ± 0.0	0.0 ± 0.0
	300	21.8 ± 0.7	12.5 ± 0.2	0.0 ± 0.0	0.0 ± 0.0
MT	0	0.6 ± 0.2	11.5 ± 0.4	7.3 ± 0.1	7.4 ± 0.2
	100	13.3 ± 0.4	9.5 ± 0.4	17.2 ± 0.1	30.9 ± 0.7
	200	24.0 ± 0.3	14.5 ± 0.2	19.4 ± 0.4	38.1 ± 0.5
	300	27.6 ± 0.7	15.5 ± 0.3	29.6 ± 0.4	41.8 ± 0.4
SWT	0	0.5 ± 0.1	13.0 ± 0.2	0.0 ± 0.0	0.0 ± 0.0
	100	13.7 ± 0.6	14.5 ± 0.3	0.0 ± 0.0	0.0 ± 0.0
	200	15.9 ± 0.3	15.0 ± 0.2	0.0 ± 0.0	0.0 ± 0.0
	300	21.8 ± 0.7	15.5 ± 0.2	0.0 ± 0.0	0.0 ± 0.0
S1	0	0.4 ± 0.1	9.5 ± 0.4	6.1 ± 0.3	9.6 ± 0.3
	100	13.6 ± 0.2	14.6 ± 0.5	17.8 ± 0.2	26.3 ± 0.4
	200	23.5 ± 0.4	15.1 ± 0.1	24.9 ± 0.1	40.5 ± 0.9
	300	32.7 ± 0.7	17.5 ± 0.2	27.2 ± 0.3	44.1 ± 0.4
S2	0	0.6 ± 0.1	7.6 ± 0.2	8.8 ± 0.2	8.5 ± 0.2
	100	19.1 ± 0.1	13.7 ± 0.1	19.4 ± 0.1	33.4 ± 0.6
	200	24.5 ± 0.1	16.9 ± 0.5	21.7 ± 0.5	43.3 ± 0.4
	300	27.8 ± 0.7	17.4 ± 0.2	26.0 ± 0.4	45.3 ± 0.4

Values are means of five replicates ± standard deviation.

the transgenic plants (**Table 1**). The S2 transgenic line had the highest dry weight, followed by the S1 line at the same salt concentration (**Table 1**).

Na^+ concentration in the leaves were measured under different salinity conditions. As shown in **Table 2**, the Na^+ concentration increased with increasing NaCl concentration, and the increase was greater in the transgenic than in the non-transgenic plants. At a NaCl concentration of 300 mM, transgenic plant leaves from line S1 had the highest Na^+ concentration, followed by those from line S2 and then line MT. The K^+ concentration increased slightly with increasing NaCl concentration (**Table 3**). These results suggest that S1 plants are able to maintain a higher osmotic potential in the cells due to the increase in the osmoticum concentration, leading to enhancement of the ability to tolerate salt stress. These results are in agreement with the finding of Alarcon *et al*. (1994) and those reported by Moghaieb *et al*. (2000).

The ectoine concentration increased with increasing salt concentration in the medium (up to approximately 45 µmol g^{-1} FW (**Table 3**). The ectoine concentrations were consistently higher in the roots than in the leaves this restriction might results from post-transcriptional regulation of the ectoine biosynthetic genes under salt stress. These results are consistent with our previous findings concerning post-transcriptional regulation of the *BADH* gene under salt stress (Moghaieb *et al.* 2000). The obtained data are also consistent with the findings of Hayashi *et al.* (1997), who reported that when *Arabidopsis thaliana* was transformed with the *cod A* gene under the control of the CaMV35S promoter, the transgenic plant was able to accumulate betaine. The ectoine concentrations in our transgenic tomato plants are much higher than the concentrations found in transgenic tobacco cell lines (70 nmol g^{-1} FW) constructed by Nakayama *et al.* (2000), and are equivalent to or higher than the glycine betaine concentrations found in transgenic maize (17 µmol g^{-1} FW; Yang *et al.* 1995).

It has been claimed that proline and betaine merely maintain cell turgor in high-osmolarity media, and are compatible with normal cellular function at high intercellular concentrations. The intercellular concentration of ectoine has been found to reach 158 mM in nonhalophilic *Streptomyces* bacteria (Malin and Lapidot 1996), and as high as 2.25 M in the halophilic bacterium *H. elongata*

(Wohlfarth 1990), whereas betaine can be synthesized to a concentration of 0.6 M in *Methanohalophilus* strain Z7401 (Lai *et al.* 1991). At high concentrations, proline, betaine and ectoine have pronounced destabilizing effects on DNA *in vitro* (Rees *et al.* 1993).

It is well known that osmotic adjustment involves the net accumulation of solutes in a cell in response to salinity, and consequently, the osmotic potential decreases, which in turn attracts water into the cells and enables the turgor to be maintained. Alarcon *et al.* (1994) had observed a direct relationship between the degree of the saline stress applied and the decrease in water stress as evidenced by the decrease of leaf turgor pressure in tomato plants.

According to Neuman *et al.* (1988), a positive ψ_p is required for cell elongation and stomatal opening. The present data indicated that the ψ_w of the salt-treated plants decreased with increasing NaCl concentrations (**Table 2**). The decrease was more pronounced in the transgenic line MT. The osmotic adjustment (OA) value increased with increasing NaCl concentrations; the increase was greater in transgenic plants than in non-transgenic plants especially at 300 mM NaCl treatment (**Table 2**). The present data indicated that the ψ_p values of the transgenic plants increased with increasing salt concentration due to accumulation of Na^+ and ectoine. The increase in ψ_p values may be responsible for the promotion of transgenic plant growth under salt stress conditions. These findings agree with our previous results regarding the transformation of tomato plants with the betaine aldehyde dehydrogenase gene and also our findings in ectoine-transformed tobacco plants (Moghaieb *et al.* 2000, 2006).

Taken together, these results indicate that ectoine synthesis is stimulated in leaves and roots by the application of salt, which improves water status by maintaining higher activities of water uptake and transport to leaves. The present study revealed that ectoine plays an important role as an osmoticum to maintain high moisture content in leaves, and to open stomata under saline conditions, leading to enhanced transpiration. Consequently, the transpiration of water through the stomata stimulates translocation of water through the xylem from the roots.

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