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Physiological and Growth Responses of Tomato Progenies Harboring the Betaine Alhyde Dehydrogenase Gene to Salt Stress

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Abstract

The responses of five transgenic tomato (*Lycopersicon esculentum* Mill) lines containing the betaine aldehyde dehydrogenase (BADH) gene to salt stress were evaluated. Proline, betaine (N, N, N-trimethylglycine, hereafter betaine), chlorophyll and ion contents, BADH activity, electrolyte leakage (EL), and some growth parameters of the plants under 1.0% and 1.5% NaCl treatments were examined. The transgenic tomatoes had enhanced BADH activity and betaine content, compared to the wild type under stress conditions. Salt stress reduced chlorophyll contents to a higher extent in the wild type than in the transgenic plants. The wild type exhibited significantly higher proline content than the transgenic plants at 0.9% and 1.3% NaCl. Cell membrane of the wild type was severely damaged as determined by higher EL under salinity stress. K^+ and Ca^{2+} contents of all tested lines decreased under salt stress, but the transgenic plants showed a significantly higher accumulation of K^+ and Ca^{2+} than the wild type. In contrast, the wild type had significantly higher Cl^- and Na^+ contents than the transgenic plants under salt stress. Although yield reduction among various lines varied, the wild type had the highest yield reduction. Fruit quality of the transgenic plants was better in comparison with the wild type as shown by a low ratio of blossom end rot fruits. The results show that the transgenic plants have improved salt tolerance over the wild type.

Key words: betaine aldehyde dehydrogenase activity; betaine contents; salt tolerance; tomato; transgenic plants.

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Excessive salinity is one of the most important edaphic factors that restricts the distribution of plants in certain natural habitats, and constitutes an increasingly severe agricultural problem in wide areas of the world (Perez Alfocea et al. 1993). Deleterious effects of saline conditions on plant growth, in general, are attributed to two main factors: reduction in osmotic potential of the rooting medium and specific ion toxicity (Eaton 1942). Osmotic adjustment by accumulation of compatible solutes, which include sugars, glycerol, amino acids, sugar alcohols and other low molecular weight metabolites, is one of the mechanisms evolved by plants to overcome saline stress (Morgan 1984; Kuznetsov and Shevyakova 1997; Yeo 1998; Verslues et al. 2006).

Betaine, which regulates osmotic pressure and protects enzyme activities, is one of the most frequently documented compatible solutes in many different organisms (Rhodes and Hanson 1993). In higher plants, betaine is synthesized by two steps of oxidation from choline via betaine aldehyde. The first step is catalyzed by choline monooxygenase (CMO, E.C. 1.1.1.) and the second by betaine aldehyde dehydrogenase (BADH, E.C.1.

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2.1.8.). Genes encoding for the betaine biosynthetic enzymes were well characterized in many plant species, including *Spinacia oleracea* (Weretilnyk and Hanson 1990), *Beta vulgaris* (McCue and Hanson 1992), *Atriplex hortensis* (Xiao et al. 1995). However, some plant species, such as tomato, *Arabidopsis*, and tobacco, are betaine non-accumulators (Weretilnyk et al. 1989; Rhodes and Hanson 1993; Nuccio et al. 1998). Furthermore, most crops, such as tomato, are sensitive to salinity at all developmental stages, leading to substantially reduced yields under salt stress (Maas 1986; Bolarín et al. 1993; Cuartero and Fernández-Muñoz 1999; Foolad 1999). The simplicity of the betaine biosynthetic pathway has attracted a great effort in the metabolic engineering of betaine accumulation as an approach to enhance salt tolerance of sensitive species (Le Rudulier et al. 1984; Guo et al. 2000; Li et al. 2000; Chen and Murata 2002).

Proline is another widely studied compatible solute for plants grown under stress conditions (Delauney and Verma 1993), which is important in osmotic adjustment in plant tissues (Stewart and Larher 1980; Hare and Cress 1997) and is also a nitrogen source during recovery from stress (Trotel et al. 1996). However, proline accumulation appeared to be merely a result of salt stress damage rather than an indication of salt tolerance (Hanson et al. 1977; Ferreira et al. 1979; Lutts et al. 1996b; Marcum 1999; De Lacerda et al. 2003; Claussen 2005). In cassava (*Manihot esculenta*), Alves and Setter (2004) found an increased concentration of proline in response to water stress, but the contribution of this increment to the total change in osmotic potential was insignificant. In tomato, negative or no correlation between proline accumulation and salt tolerance was detected (Shannon et al. 1987; Alian et al. 2000).

Previously, most of the published research with improved salt tolerance of transgenic plants was largely based on characterization of some physiological parameters, without taking yield into account (Flowers 2004). In addition, the characterization of transgenic tomato progenies under salt stress is not widely reported.

We have reported the transformation of tomato cultivar Bailichun with the *BADH* gene (X68770 in EMBL Data Library, and the patent no: ZL97125830.9) from *A. hortensis* (Jia et al. 2002). The T0 generation of transgenic tomato plants showed significantly higher levels of BADH activity than the wild type under salt stress. Analyses of root development and relative electronic conductivity indicated that the transgenic plants exhibited salt tolerance up to 120 mmol/L. To further characterize salt tolerance of the following generations, five homozygous tomato progenies were studied. Besides the BADH activity, betaine, proline, ion and chlorophyll contents, electrolyte leakage (EL), the growth parameters during salt stress were also evaluated in comparison with the wild type.

Results

Integration and expression of the *BADH* gene in transgenic tomato

Using specific polymerase chain reaction (PCR) primers, the presence of the *BADH* gene was detected in all the transgenic plants, while this gene was not detected in the wild type (Figure 1). To analyze the function of the foreign gene, BADH activity and betaine content were measured in the transgenic progenies and are summarized in Table 1. The enzyme activity and betaine content were almost undetectable in the wild type under either normal or stressed conditions. The transgenic plants exhibited low levels of BADH activity under normal conditions, while the enzyme activity increased up to 20-fold when stressed by 1.0% and 1.5% NaCl. The BADH activity varied in different transgenic lines. Line T3-5 had the highest value of BADH activity under 1.5% NaCl stress. Under salt stress, all genetically modified plants had significantly higher betaine contents than the wild type, although lines T3-1, T3-3 and the wild type showed similarly marginal betaine contents under control conditions. The transgenic plants showed 10- to 24-fold increases in betaine contents under salt stress and the highest amount of betaine content under 1.0% and 1.5% NaCl stresses occurred in lines T3-8 and T3-5. In contrast, the wild type had a negligible accumulation of betaine.

Ion content and electrolyte leakage

K^+ and Ca^{2+} contents of the plants decreased with the increase of NaCl concentration in the irrigating solution (Table 1). However, the decrease was less in the transgenic plants compared to the wild type. In salinized conditions, K^+ and Ca^{2+} contents of the transgenic plants were significantly higher than

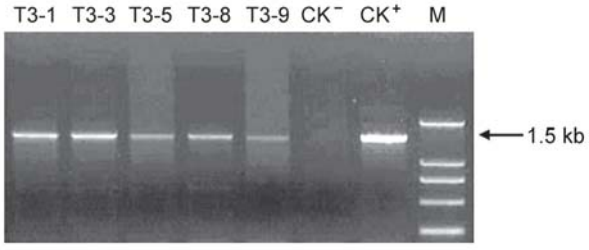

Figure 1. Polymerase chain reaction (PCR) analysis of cv. Bailichun wild type (WT) and the transgenic plants (T3-1, T3-3, T3-5, T3-8, T3-9).

M, molecular-weight ladder; CK^+, positive control, the plasmid DNA of pBin438; CK^-, negative control, genomic DNA from a non-transformed wild type plant.

Table 1. Betaine aldehyde dehydrogenase (BADH) activity (nmol·min^{-1}·mg^{-1} protein), betaine contents (μmol/g DW) and ion contents (mg/g DW) of cv. Bailichun wild type (WT) and the transgenic plants (T3-1, T3-3, T3-5, T3-8, T3-9) under different NaCl stresses

Parameters measured	NaCl level (%)	Lines					
		T3-1	T3-3	T3-5	T3-8	T3-9	WT
BADH activity	0	1.11 ± 0.10a	0.86 ± 0.12b	0.45 ± 0.11c	0.78 ± 0.19b	1.19 ± 0.21a	0.14 ± 0.09d
	1.0	6.35 ± 0.25d	7.84 ± 0.36b	7.23 ± 0.45c	8.26 ± 0.51a	7.36 ± 0.28c	0.17 ± 0.12e
	1.5	5.23 ± 0.11e	6.62 ± 0.36b	8.81 ± 0.41a	6.25 ± 0.45c	5.78 ± 0.37d	0.15 ± 0.11f
Betaine contents	0	0.23 ± 0.16bc	0.22 ± 0.18c	0.45 ± 0.21a	0.43 ± 0.19a	0.31 ± 0.21b	0.16 ± 0.15c
	1.0	5.52 ± 0.22b	5.34 ± 0.67b	4.98 ± 0.44c	6.23 ± 0.35a	3.97 ± 0.39d	0.21 ± 0.20e
	1.5	4.41 ± 0.19b	5.11 ± 0.36b	7.36 ± 0.54a	4.16 ± 0.68c	3.57 ± 0.46d	0.19 ± 0.18e
K^+	0	48.5 ± 2.96a	48.8 ± 2.03a	52.0 ± 0.74a	49.0 ± 1.93a	48.9 ± 1.40a	49.7 ± 1.54a
	1.0	40.8 ± 2.00bc	38.5 ± 3.25c	47.2 ± 1.18a	44.1 ± 2.16ab	36.9 ± 2.36c	30.7 ± 1.19d
	1.5	37.7 ± 2.02b	36.5 ± 2.76b	42.4 ± 3.42a	36.9 ± 2.48b	36.4 ± 2.66b	27.3 ± 1.77c
Ca^{2+}	0	52.5 ± 2.86a	51.7 ± 1.95a	51.1 ± 1.25a	52.3 ± 2.91a	51.8 ± 1.91a	51.9 ± 2.25a
	1.0	45.9 ± 1.71ab	46.0 ± 3.76ab	40. 9 ± 1.94b	48.5 ± 1.91a	45.2 ± 1.4ab	34.6 ± 2.01c
	1.5	40.0 ± 3.25ab	36.2 ± 3.24b	40. 1 ± 1.99a	41.9 ± 1.63a	41.2 ± 3.07a	28.8 ± 1.65c
Na^+	0	2.5 ± 0.15a	2.7 ± 0.20a	2.7 ± 0.15a	2.5 ± 0.17a	2.6 ± 0.10a	2.7 ± 0.10a
	1.0	44.2 ± 1.96b	43.9 ± 1.71b	42.2 ± 1.12bc	40.6 ± 1.51c	42.3 ± 1.00bc	64.5 ± 0.81a
	1.5	58.0 ± 2.37c	59.8 ± 3.37bc	62.9 ± 1.90b	61.7 ± 3.44bc	62.7 ± 2.64b	86.1 ± 1.37a
Cl^-	0	7.9 ± 0.25a	8.1 ± 0.21a	7.9 ± 0.25a	7.7 ± 0.29a	8.1 ± 0.29a	8.0 ± 0.35a
	1.0	53.5 ± 1.16bc	55.8 ± 3.24b	51.8 ± 1.84bc	52.1 ± 3.19bc	50.9 ± 1.53c	66.0 ± 1.67a
	1.5	84.5 ± 3.65b	83.9 ± 1.64bc	84.8 ± 1.30b	84.9 ± 4.48b	78.6 ± 1.11c	106.5 ± 5.11a

Means followed by the same letter in each line for the same salt treatment do not differ statistically at 5% probability, by least significant difference (LSD) multiple comparisons. Values are the means of three replicates ± *SE*.

those of the wild type. While in normal conditions, no difference was detected. High levels of K^+ contents (37–42 mg/g dry weight DW) were detected in the transgenic plants grown in 1.5% NaCl compared to the wild type control (27 mg/g DW). Compared to the normal irrigation, the transgenic plants grown under 1.5% NaCl had a Ca^{2+} reduction of 20–30%, whereas the reduction in the wild type was as high as 45%. The wild type had significantly higher Na^+ and Cl^- accumulation than the transgenic plants in stress conditions. The Na^+ contents of the wild type were 64.5 and 86.1 mg/g DW under 1.0% and 1.5% NaCl stress, respectively, but the highest values of the corresponding transgenic plants were 42.9 and 62.9 mg/g DW. Under 1.0% and 1.5% NaCl stress, the Cl^- contents of the wild type were 25.0% and 27.8% higher than the corresponding average of the transgenic plants. These results indicate that the transgenic plants have a higher K^+ and Ca^{2+} accumulation and lower Cl^- and Na^+ accumulation than the wild type under saline conditions.

For all lines, low values of EL were recorded under normal conditions (Figure 2). EL increased with the increase of salt concentration and reached the maximum value at 1.5% NaCl. The presence of 1.5% NaCl in the growth medium resulted in over a two-fold increase of EL in the wild type compared with that under control condition. Line T3-5 had the least EL increase at both salt concentrations, indicating a stronger ability to withstand salt stress.

Proline content

Figure 3 shows that the wild type had higher proline accumulation than the transgenic plants under salinized conditions. Proline contents increased with the increase of NaCl concentration in the growth medium when NaCl was below 0.7%. The

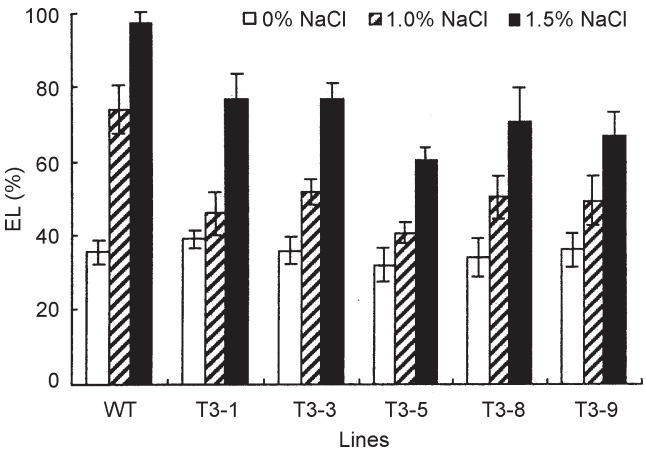

Figure 2. Electrolyte leakage (EL) in cv. Bailichun wild type (WT) and the transgenic plants (T3-1, T3-3, T3-5, T3-8, T3-9) under different NaCl stresses.

Values are the means of four replicates ± *SE*.

wild type showed a distinct higher proline content than the transgenic plants at 0.9% and 1.3% NaCl. No difference was detected between the wild type and the transgenic plants under 1.5% NaCl. It was noteworthy that the highest proline content of the wild type was 4.36 μmol/g FW (fresh weight), while that of the corresponding value in the transgenic plants was 2.89 μmol/g FW.

Pigment contents

As shown in Figure 4, when the salt level was higher than 0.3%, the chlorophyll contents decreased except for lines T3-8 and T3-9, mostly due to a strong reduction in chlorophyll *a* content and to a lesser extent reduction in chlorophyll *b* content (Data not shown). However, the wild type had a pronounced decrease compared to the transgenic plants as manifested by its more salinity-induced chlorotic spots and necrosis in the old leaves. The Pearson correlation coefficient between salt level and chlorophyll contents was –0.832 ($P < 0.01$). At the lower salt level the chlorophyll contents increased with the salt level; it seems that a relatively lower salt stress could stimulate the chlorophyll synthesis in salinized plants.

Blossom end rot fruits

Blossom end rot (BER) fruits of all the tomato plants increased with increasing NaCl concentration (Figure 5). But the increase was more obvious in the wild type than in the transgenic plants. The wild type plants had more BER fruits than the transgenic plants at both salt treatments. The ratio of BER fruits in the wild type was 10.5% and 13.8% at 1.0% and 1.5% NaCl, respectively. In contrast, the average BER ratio of the transgenic lines was 6.1% and 6.7%. Line T3-8 had the least ratio of BER fruits at 1.0% and 1.5% NaCl stress, which was 4.3% and 4.9%, respectively. The results indicated that the transgenic lines had an improved fruit quality compared to the wild type under salinity condition.

Growth parameters

Although a slight decrease was seen in the stem diameter under salinity, there was no significant difference among the treatments (Table 2). Only 1.5% NaCl reduced the internode numbers, but no significant difference was observed in lines T3-5 and T3-8. Leaves per plant and plant height followed the same pattern as internode number, except line T3-9, which did not change significantly under 1.0% and 1.5% NaCl stresses. Salt stress significantly reduced yield per plant in both wild type and transgenic plants, but the decrease was less obvious

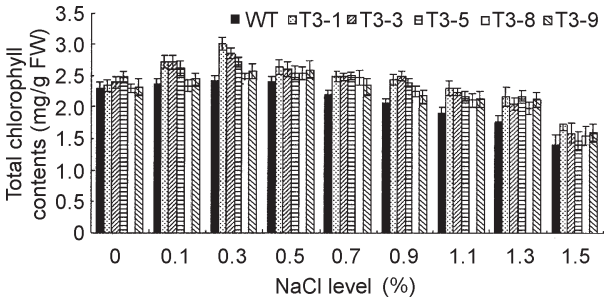

Figure 4. Total chlorophyll contents (mg/g FW) in cv. Bailichun wild type (WT) and the transgenic plants (T3-1, T3-3, T3-5, T3-8, T3-9) under different NaCl stresses.

Values are the means of four replicates ± *SE*.

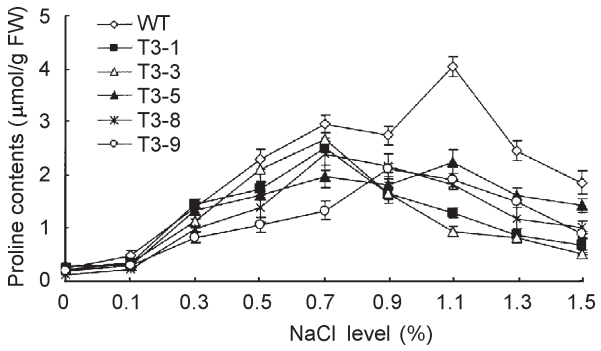

Figure 3. Proline contents (μmol/g FW) in cv. Bailichun wild type (WT) and the transgenic plants (T3-1, T3-3, T3-5, T3-8, T3-9) under different NaCl stresses.

Values are the means of four replicates ± *SE*.

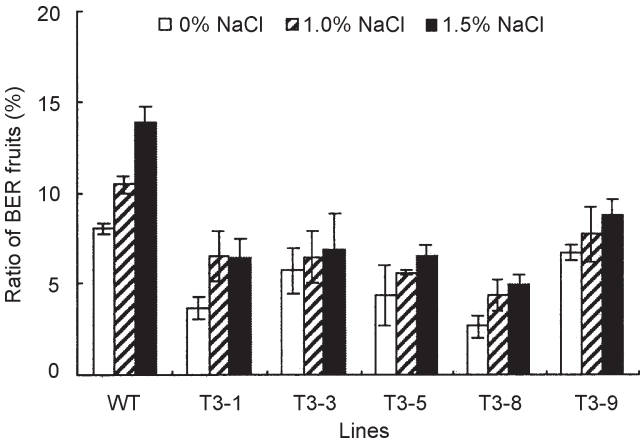

Figure 5. Ratio of Blossom end rot (BER) fruits in cv. Bailichun wild type (WT) and the transgenic plants (T3-1, T3-3, T3-5, T3-8, T3-9) under different NaCl stresses.

Values are the means of 12 replicates ± *SE*.

Table 2. Stem diameter, internode number, leaves per plant, plant height, and yield per plant of cv. Bailichun wild type (WT) and the transgenic plants (T3-1, T3-3, T3-5, T3-8, T3-9) under different NaCl stresses

Lines	NaCl level (%)	Stem diameter (cm)	Internode number	Leaves/ plant	Plant height (cm)	Yield/ plant (g)
T3-1	0.0	1.9 ± 0.21a	30.3 ± 4.50a	31.9 ± 4.32a	192.4 ± 31.27a	1 402.4 ± 147.06a
	1.0	1.8 ± 0.21a	28.0 ± 3.44ab	30.2 ± 3.74ab	176.3 ± 22.84a	778.4 ± 88.07b
	1.5	1.7 ± 0.24a	25.3 ± 2.70b	27.5 ± 2.75b	149.4 ± 10.46b	750.1 ± 67.71b
T3-3	0.0	1.9 ± 0.35a	31.2 ± 3.93a	32.3 ± 4.00a	194.6 ± 27.26a	1 450.8 ± 166.15a
	1.0	1.8 ± 0.15a	27.5 ± 3.15b	29.5 ± 2.88b	180.9 ± 16.33a	826.3 ± 81.60b
	1.5	1.7 ± 0.23a	25.8 ± 2.99b	28.2 ± 2.66b	163.3 ± 10.77b	788.0 ± 76.18b
T3-5	0.0	2.0 ± 0.15a	29.4 ± 4.17a	31.4 ± 5.58a	191.5 ± 36.98a	1 189.8 ± 163.18a
	1.0	1.8 ± 0.16a	26.7 ± 1.51a	29.6 ± 2.78ab	188.8 ± 16.9a	796.3 ± 84.01b
	1.5	1.7 ± 0.31a	25.8 ± 1.99a	26.5 ± 2.84b	167.9 ± 14.95b	678.9 ± 75.54c
T3-8	0.0	1.9 ± 0.18a	31.1 ± 5.88a	30.9 ± 4.14a	200.3 ± 2.00a	1 626.2 ± 146.55a
	1.0	1.7 ± 0.14a	27.8 ± 2.52a	28.2 ± 1.59b	185.1 ± 7.65b	890.1 ± 85.24b
	1.5	1.6 ± 0.19a	24.6 ± 2.97a	28.1 ± 2.11b	170.3 ± 13.13c	851.1 ± 89.37b
T3-9	0.0	1.8 ± 0.24a	30.1 ± 5.84a	30.5 ± 5.7a	201.8 ± 26.82a	1 612.8 ± 148.91a
	1.0	1.7 ± 0.16a	26.2 ± 1.47b	28.0 ± 1.86a	180.0 ± 14.81b	896.5 ± 84.34b
	1.5	1.6 ± 0.15a	26.2 ± 5.29b	28.2 ± 3.1a	171.1 ± 17.4b	820.3 ± 98.16b
WT	0.0	1.9 ± 0.18a	27.8 ± 5.51a	28.8 ± 5.25a	201.3 ± 43.34a	1 360.9 ± 106.42a
	1.0	1.8 ± 0.18a	26.3 ± 2.34a	27.5 ± 2.24ab	181.9 ± 14.9a	665.3 ± 84.85b
	1.5	1.7 ± 0.24a	23.0 ± 2.89b	25.3 ± 3.11b	153.3 ± 12.53b	598.3 ± 95.12b

Means followed by the same letter in columns for each line do not differ statistically at 5% probability, by least significant difference (LSD) multiple comparisons. Values are the means of 12 replicates ± *SE*.

in the transgenic plants. No significant difference in yield was found in most lines between the 1.0% and 1.5% NaCl treatment, except line T3-5. Under 1.0% and 1.5% NaCl treatment, the wild type had the yield per plant of 665.3 g and 598.3 g, accounting for 80.0% and 76.8% of the relevant average of the transgenic plants, respectively. The relative yield reduction per plant varied in different lines and different salt concentrations (Table 3). The wild type had the highest yield reduction of 51.1% and 56.0% under 1.0% and 1.5% NaCl, respectively. While line T3-5 had the least yield reduction of 33.1% and 42.9%.

Discussion

Our results demonstrate a distinct difference in response to salt stress between the transgenic plants and the wild type. The transgenic plants have a higher accumulation of betaine, K^+ and Ca^{2+} under salt treatment. However, the wild type have a higher accumulation of Cl^-, Na^+ and proline. The betaine accumulation in transgenic plants plays an important role for their improved performance, as was evidenced by higher yields than the wild type under salinity stress.

Intracellular betaine concentration in salinized plants had a positive correlation with stress tolerance (Ishitani et al. 1993; Kishitani et al. 1994; Nomura et al. 1995; Xu et al. 2001). By transforming tomato with the gene responsible for betaine synthesis, Park et al. (2004) found that the enhanced seed germination after stress conditions was significantly correlated with the amount of betaine accumulated in the transgenic plants. In the current study, the PCR analysis (Figure 1) and the BADH enzyme assay (Table 1) showed that the *BADH* gene had been stably inherited to the T3 generation. The higher betaine accumulation in the transgenic plants corresponds to its enhanced salt tolerance (Table 1). In contrast, almost no betaine was detected in the wild type under either stress or normal conditions. However, the betaine content in the transgenic plants was relatively low (<8 μmol/g DW) compared with stressed betaine accumulators (4–40 μmol/g DW) (Rhodes and Hanson 1993), apparently insufficient for significant contribution to intercellular osmotic potential adjustment. Similar results were also obtained in transgenic tobacco (Nuccio et al. 2000; Holmström et al. 2000), rice (Sakamoto et al. 1998; Su et al.

Table 3. Relative yield reduction per plant of cv. Bailichun wild type (WT) and the transgenic plants (T3-1, T3-3, T3-5, T3-8, T3-9) compared with the control

NaCl level	Relative yield reduction (%)					
	T3-1	T3-3	T3-5	T3-8	T3-9	WT
1.0%	44.5	43.0	33.1	45.3	44.4	51.1
1.5%	46.5	45.7	42.9	47.7	49.1	56.0
Average	45.5	44.4	38.0	46.5	46.8	53.6

2006), and *Arabidopsis* (Hayashi et al. 1997; Sakamoto et al. 2000). Previously, it was proposed that stress tolerance conferred by betaine accumulation in transgenic plants might be due to its protective function in stabilizing the quaternary structures of enzymes and in maintaining membrane structure at high salt concentrations or at extreme temperatures (Papageorgiou and Murata 1995; Chen and Murata 2002; Yang et al. 2005), rather than its osmotic function. Most recently, Ohnishi and Murata (2006) showed that betaine protected PSII against photoinhibition in *Synechococcus* under 220 mmol/L NaCl stress. In this study it is likely that the protective role of betaine in the transgenic tomatoes could at least partially mitigate the damage on the enzymes caused by excess toxic ions, which are correlated with previous results in wheat (Guo et al. 2000) and tobacco (Xiao et al. 1995). It was also reported that exogenous betaine application to plants had beneficial effects against various stresses and increased plant yields under salt conditions (Mäkelä et al. 1998; Díaz-Zorita et al. 2001; Xu et al. 2001; Park et al. 2004; Yang and Lu 2005).

In several species the absorption of relatively high amounts of Cl^- and/or Na^+ in their expanded leaves were the primary causes responsible for their salt sensitivity (Greenway and Munns 1980). This was mostly caused by inadequate cellular compartmentation in their leaves. In our research, the wild type had significantly higher toxic ion accumulation than the transgenic plants under salinity stress (Table 1). This is in agreement with the relatively higher EL (Figure 2) of the wild type in salinized conditions, because excessive Cl^- and Na^+ ions could exert their adverse effect on membrane permeability (Greenway and Munns 1980). Jolivet et al. (1983) reported interactions of betaine with ion fluxes in beet tissue. Moreover, exogenously-supplied betaine in rooting medium could decrease root and shoot Na^+ contents in barley (Ahmad et al. 1987). Considering the lower toxic ion contents and the higher betaine accumulation in the transgenic plants, it was reasonable to hypothesize that betaine was most likely to have an important role in modifying membrane transport properties in addition to its function as a compatible solute. Excessive Na^+ could also have detrimental effect on K^+ acquisition and nutrition (Rus et al. 2004). The higher concentration of K^+ in the leaves of transgenic plants exposed to salinity (Table 1) may be beneficial for maintaining higher stomatal K^+, as suggested by Chow et al. (1990). The higher Ca^{2+} accumulation in the transgenic plants might decrease salt damage. Previous data showed that Ca^{2+} is important in controlling K^+/Na^+ selectivity, which reduces Na^+ uptake and increases NaCl tolerance (Demidchik et al. 2002; Zhu 2003). In addition, a reduction in Ca^{2+} uptake for tomatoes grown in saline conditions usually leads to the appearance of BER (Adams and Ho 1992; Cuartero and Fernández-Muñoz 1999). Our result on the relative higher Ca^{2+} concentration and the corresponding less BER in the transgenic plants (Figure 5) agrees with the previous conclusion.

The wild type exhibited a higher ability of proline accumulation than the transgenic plants (Figure 3). Taking the yield reduction and the proline level in different lines into consideration, it seems that the increase of proline contents in salinized tomatoes was most likely the result of salt damage. Therefore, it is possible that the higher betaine accumulation in the transgenic tomatoes protects the plants from salt damage, at least to some extent, thus reducing their proline levels. It has been reported that the exogenous application of 1 mmol/L betaine on 135 mmol/L NaCl stressed tomato significantly decreased the proline contents (Heuer 2003). Application of abscisic acid induces proline but not betaine accumulation in barley leaves, indicating that the biochemical signal(s) regulating the accumulation of betaine and proline was different (Pesci and Reggiani 1992). However, no published work on the relationship between proline and betaine in transgenic plants has been documented. Further study is required to elucidate the exact mechanism.

The reduction of productivity in many plants subjected to excessive salinity is often associated with reduced photosynthetic capacity as determined by lower chlorophyll contents (Sultana et al. 1999; Netondo et al. 2004). Under salt stress, the reduction of chlorophyll contents in the wild type was more obvious than that in the transgenic plants (Figure 4), showing a more severe salt damage as evidenced by more brownish leaves and visible loss of turgor. The increased activity of the chlorophyll degrading enzyme or the suppression of the specific enzyme responsible for pigments synthesis may attribute to the decreased chlorophyll contents in salt-stressed plants (Strogonov 1973; Reddy and Vora 1986).

Although salt stress had different influences on the internode number, leaves per plant and plant height (Table 2), no significant correlation between these parameters and the yield per plant was detected (Data not shown). The wild type had the most reduction in yield per plant under both salt treatments (Table 3). Based on the results on ion contents, betaine accumulation and yield reduction, the transgenic plants had acquired improved salt tolerance with respect to the wild type, mainly due to the betaine accumulation.

Materials and Methods

Plant material and growth conditions

Seeds from T2 generation (lines T2-1, T2-3, T2-5, T2-8 and T2-9) of transgenic tomato containing the *BADH* gene were germinated in vermiculite and grown in a growth chamber at 25/20 °C (day/night) temperature with a relative humidity of 50 ± 10%. The wild type cv. Bailichun was used as control. Ten days after rooting, young seedlings were transferred to the half-strength Hoagland nutrient solution (Ostrem et al. 1987) until

the five-leaf-stage. Then individuals of similar height were picked out and transplanted to three bottom-perforated tanks (10 m × 4 m × 0.5 m) filled with the growing medium composed of vermiculite, turf and humus (1:1:1, v:v:v) in a greenhouse. Seedlings were allowed to grow for 4 weeks before NaCl treatment. The plants were exposed to salinity by adding NaCl to the growth medium in 0.1% or 0.15% increment every 5 d, until the final concentrations of 1.0% or 1.5% were reached. Then the plants were irrigated twice with NaCl solution of the final concentration. The half-strength Hoagland nutrient solution (Ostrem et al. 1987) used for irrigation added with or without NaCl was stored in three 1 000-L reservoirs. The salt concentrations were determined by measuring the electrolyte conductance of the leachates. The top fully expanded leaf was cut off on the fourth day of each irrigation interval and was used for the analysis of proline and chlorophyll contents. EL, BADH enzymatic activity, betaine and ion contents were assayed using the leaves of the same type as for the proline analysis on the fourth day of salt levels amounting to 1.0% and 1.5%.

The day/night temperature in the greenhouse ranged from 25 to 33 °C and 18 to 23 °C, respectively. Humidity ranged from 40 to 85%. Light averaged at 1 050 $\mu mol \cdot m^{-2} \cdot s^{-1}$ with a minimum of 200 and a maximum 1 400 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at noon. Four replicates were arranged in a completely randomized design.

DNA extraction and polymerase chain reaction

Total genomic DNA was isolated from both the wild type and the transgenic plants according to a method described by Murray and Thompson (1980). The PCR procedure was carried out as follows. DNA (0.5 μL) was added to a final volume of 20 μL with 0.2 μmol/L of each primer, 0.2 mmol/L each of dNTP, and 1 U *Taq* DNA polymerase. The reaction consisted of 35 cycles of 1.0 min at 94 °C for denaturation, 1.0 min at 55 °C for annealing, and 1.5 min at 72 °C for extension. The two primers for *Atriplex hortensis BADH* gene were: 5'AGAATGGCGTTCCCAATTCCTGCTC-3' and 5'TTCAAGGAGACTTGTACCATCCCCA-3' (Xiao et al. 1995).

Proline and chlorophyll contents

Proline content was measured following the method of Bates et al. (1973). Two grams of plant material was homogenized in 3% aqueous sulfosalicylic acid and centrifuged at 10 000*g*. The supernatant was used for the measurement of proline content.

For chlorophyll analysis, six discs punched from the top fully expanded leaves were used. Pigments were extracted in 80% (v/v) acetone/water and the total chlorophyll contents were determined using a spectrophotometer (UV-160, Shimadzu, Japan) according to Arnon (1949).

BADH activity

Activity of BADH was assayed as described by Livingstone et al. (2003). Two grams of washed leaves were ground in a mortar and centrifuged. The supernatant was kept at 37 °C for the measuring of BADH activity later. The standard assay mixture consisted of 0.5 mL 0.5 mol/L glycine-NaOH buffer (pH 8.7), 24 μL 0.1 mol/L betaine aldehyde (BAL), 0.1 mL 20 mmol/L NAD and enzyme solution to a total volume of 3 mL.

Betaine content

The extraction and purification of betaine were carried out as described by Hitz and Hanson (1980). Two grams of leaves were ground to powder and then incubated with 10 mL deionized water at 4 °C for 24 h. After centrifugation, the upper phase was dried, redissolved in 2 mL deionized water and applied to a 5-mL Dowex 1 × 8 (100–200 mesh, OH^- form, Sigma, St. Louis, USA) column. Betaine was eluted with 10 mL H_2O and the aqueous eluant was applied to a 5 mL Dowex 50 W × 2 (50–100 mesh, H^+ form, Serva, Heidelberg, Germany) column. After watering the latter column, bound betaine was recovered with 10 mL 6 mol/L NH_4OH, and the eluate was dried at 65 °C.

The high performance liquid chromatography (HPLC) system (Waters Corporation, Milford, MA, USA) was used to determine betaine concentration as described by Chen et al. (2000). Separation was carried out using a 250 mm × 4.6 mm stainless steel column (packed with 10 μm irregular-H) eluted with 50 mmol/L KH_2PO_4 containing the ion-pair agent 0.1% PIC B-8 (1-octane sulfonic acid, Waters).

Electrolyte leakage and ion content

Electrolyte leakage was assessed as described by Lutts et al. (1996a), using a HI9033 conductivity meter (Hanna, Italy). K^+, Na^+ and Ca^{2+} contents were determined according to Alian et al. (2000) in dried plant materials using a flame photometer (Corning, Essex, England). For chloride determination, dry plant material was extracted with 0.1 mol/L HNO_3 in 10% (v/v) acetic acid and Cl^- was determined using a digital chloridometer (Buchler Instruments, Kansas City, MO, USA).

Growth parameters

About 3 months after being transplanted to the greenhouse, when the plants were in their late reproductive stage, the stem diameter, internode number, plant height and leave number were recorded. If the light green to brown areas on the blossom end of a mature fruit was over 1 cm^2, the fruit was marked as a blossom end rot (BER) fruit. Yield per plant was recorded as

the accumulative output. After the growth parameters were measured, the remaining fruits were harvested together and weighed.

Statistical analysis

Besides the actual yield per plant, for standardizing data, the relative yield reduction in comparison with the control was also calculated using the following formula: relative yield reduction per plant (%) =(1–(salinized yield/control yield))×100. Analyses of variance (ANOVA) for variables from measurements were used for testing the lines and treatments differences were used. Means and standard errors were reported. The significance was tested using least significant difference (LSD) at 5%. The SPSS 10.0 (for Windows) statistical package was used for the statistical analysis.

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