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Effect of High Electrical Conductivity of Hydroponic Nutrient Solution on Vaccine Protein Content in Transgenic Tomato

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SUMMARY. Using greenhouse tomato (*Solanum lycopersicum*) as a model system to produce pharmaceutical proteins, electrical conductivity (EC) of hydroponic nutrient solution was examined as a possible factor that affects the protein concentration in fruit. Transgenic tomato plants, expressing F1-V protein, a plant-made candidate subunit vaccine against plague (*Yersinia pestis*), were grown hydroponically at high (5.4 dS·m⁻¹) or conventional EC [2.7 dS·m⁻¹ (control)] with a high-wire system in a temperature-controlled greenhouse. There was no significant difference in plant growth and development including final shoot dry weight (DW), leaf area, stem elongation rate, or leaf development rate between high EC and control. Net photosynthetic rate, transpiration rate, and stomatal conductance (g_s) of leaves were also not significantly different between EC treatments. For both EC treatments, immature green fruit accumulated DW at a similar rate, but dynamics observed in fruit total soluble protein (TSP) and F1-V during the fruit growth were different between the two ECs. Fruit TSP concentration per unit DW decreased while TSP content per whole fruit increased as fruit grew, regardless of EC. However, TSPs were significantly lower in high EC than in control. Fruit F1-V concentration per unit DW and F1-V content per whole fruit were also lower in high EC than in control. Our results found that increasing EC of nutrient solution decreased TSP including the vaccine protein in fruit, suggesting that adjusting nutrient solution EC at an appropriate level is necessary to avoid salinity stress in this transgenic tomato.

Plant-made pharmaceutical (PMP) protein production or molecular farming is attracting considerable interest as a novel system for production of therapeutic proteins. Using plant-based expression systems, various PMP proteins, including vaccines, antibodies, and other proteins such as hormones, growth factors, blood proteins, cytokines, and enzymes can be synthesized (De Muijnck et al., 2010; Matoba et al., 2011; Rybicki, 2010). Several PMP products have been or are

tested in early phase clinical trials and show safety and efficacy (Yusibov et al., 2011). The biotechnology-based pharmaceutical market is more than \$80 billion in 2008 (Strohl and Knight, 2009), and PMP may be expected to account for a part of it in the near future. Greenhouse tomato production is considered as a suitable system for PMPs in terms of availability of a relatively efficient transformation system (Mason et al., 2002), high biomass yield (Twyman et al., 2003), containment

to prevent transgene flow to the outside (Twyman et al., 2003), and capability of environmental control for steering the plant growth to maximize the protein productivity with minimum input of available resources (Matsuda et al., 2009).

Alvarez et al. (2006) developed transgenic tomato lines transformed with the *Y. pestis* *f1-v* fusion gene encoding the F1-V fusion protein, a subunit vaccine candidate against bubonic and pneumonic plague, and driven by the constitutive cauliflower mosaic virus (CaMV) 35S promoter. They demonstrated with mice that orally delivered freeze-dried fruit of the transgenic plants was immunogenic (Alvarez et al., 2006) and protective against a challenge of *Y. pestis* (Alvarez and Cardineau, 2010), suggesting that it can be used as an edible vaccine. Our previous studies showed that the transgenic plants grown in a growth chamber (Alvarez et al., 2006) had a 6-fold higher fruit F1-V protein concentration than those in a greenhouse (Matsuda et al., 2009), suggesting that the fruit F1-V protein concentrations were possibly affected by growing conditions, cultural practice, or both. However, partly because protein is not a typical quality attribute in tomato fruit, there is limited information available for environmental and cultural factors affecting fruit protein concentrations. We believe that target protein productivity can be maximized by carefully optimizing environmental conditions around the plants and that inappropriate control of abiotic environments could decrease the production of transgenic proteins in plants. In the commercial context, the optimal environments for PMP production would be indispensable information for enhancing protein productivity per unit area and time or for preventing the potential loss of a protein product.

Stevens et al. (2007) showed that moderate water stress due to less irrigation enhanced the concentration of subunit vaccine candidate against anthrax in leaves of transgenic tobacco (*Nicotiana tabacum*). Providing water stress to plants by lowering water potential in the root zone is simply, predictably, and economically achievable in greenhouse tomato hydroponics, by increasing the EC of the nutrient solution by adding sodium chloride (NaCl) (Wu and Kubota, 2008a) and has been commercially practiced to

improve the flavor in tomato (Wu and Kubota, 2008a). Applying low water potential in root zone generally “concentrates” soluble solids in tomato fruit (Adams, 1991; Krauss et al., 2006; Lin and Glass, 1999; Mitchell et al., 1991; Wu et al., 2004; Wu and Kubota, 2008b). Similarly, fruit TSP and F1-V in fruit may be increased in the transgenic tomato grown under a high EC (more negative water potential in the root zone). However, to our knowledge, there is no information available on the dynamics of TSP or a target protein concentration in tomato fruit under high EC.

The aim of this study was thus to investigate the effects of an increased EC of hydroponic nutrient solution on TSP and F1-V protein concentrations in fruit of the transgenic tomato grown in a greenhouse. Whole-plant growth and development, and leaf gas exchange characteristics were also evaluated to examine whether there were adverse effects of the high EC (Adams, 1991; Romero-Aranda et al., 2001). Results would provide information on how important strict management of nutrient solution EC is for optimizing practical F1-V production with the transgenic tomato in greenhouse.

Materials and methods

PLANT MATERIAL AND GROWTH CONDITIONS. The primary transformant (T_0) of the transgenic tomato plants expressing F1-V protein had been obtained previously by *Agrobacterium*-mediated transformation of a wild-type ‘TA234’ with the *γ. pestis fl-v* fusion

gene driven by the constitutive CaMV 35S promoter (Alvarez et al., 2006). One plant of its T_1 progeny, line 22.11, showing a substantially high F1-V expression level of 11% of fruit TSP (Alvarez et al., 2006), was selected and allowed to self-fertilize. One of its T_2 progeny, line 22.11.5, was selected based on their relatively high F1-V expression levels and on their visual normality, and its cuttings were vegetatively propagated to ensure a genetically homogenate population and were used in the experiment.

After rooted and acclimatized, the plants were grown hydroponically with a high-wire system in an acrylic, Biosafety-Level-2 greenhouse equipped with evaporative fan-and-pad cooling system in Tucson, AZ, from July to Oct. 2008. Cultivation conditions were described in detail by Matsuda et al. (2010). Briefly, a mixture of 1 perlite:1 vermiculite (by volume) was used as a growth media, placed in 2-gal black plastic pots. A nutrient solution was supplied using a drip irrigation system. The basal composition of the full-strength modified Hoagland nutrient solution was prepared according to Wu and Kubota (2008b) with slight modifications (Matsuda et al., 2009) except that the concentration of nitrogen, derived from potassium nitrate and calcium nitrate, was 100 mg·L⁻¹. The irrigation volume per application, irrigation frequency, and daily irrigation period when the irrigation took place at the designated irrigation frequency were increased depending on plant growth so as to maintain a minimum of 30% efflux of the nutrient solution and to avoid nutrient accumulation: from 100 to 200 mL, from once per 60 min to once per 20 min, and from 7.5 to 9 h·d⁻¹, respectively. Mean daytime and nighttime air temperatures

during the experiment were about 24 and 22 °C, respectively. Daily photosynthetic photon flux (PPF) integral on sunny days was between 15 and 20 mol·m⁻²·d⁻¹ and that on average over the experimental period was about 18 mol·m⁻²·d⁻¹. Trusses below the ninth node were removed before anthesis. Fruit were pruned to seven per truss.

TREATMENTS. On 30 Aug. 2008, when the first trusses above the ninth node were allowed to flower, EC treatments started. The high EC (5.4 ± 0.3 dS·m⁻¹) was achieved by adding 1.6 g·L⁻¹ NaCl to the basic nutrient solution described above. Nutrient solution for the control plants was unamended (2.7 ± 0.2 dS·m⁻¹). The high EC of 5.4 dS·m⁻¹ was determined because a moderately high EC of around 5 dS·m⁻¹ reportedly increased fruit soluble solids without decreasing leaf photosynthesis and yields in wild-type tomato (Leonardi et al., 2004; Wu and Kubota, 2008a, 2008b). The EC levels of the nutrient solution tanks were monitored and adjusted if necessary once a day according to the nutrient recipe in each treatment. Six plants were subjected to each treatment.

GAS EXCHANGE MEASUREMENTS. Measurements of net photosynthetic rate (P_n), transpiration rate and g_s in young, fully expanded leaves were carried out using a portable photosynthesis system equipped with a halogen light source (CIRAS-2; PP Systems, Amesbury, MA) between 5 and 7 Oct. 2008. Measurements were made under saturating light conditions at a PPF of 1500 μmol·m⁻²·s⁻¹, an atmospheric carbon dioxide concentration of 340 ± 10 ppm which was close to that inside the greenhouse, a leaf temperature of 28 °C, and a leaf-to-air vapor pressure deficit of 1.2 ± 0.2 kPa.

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Units

To convert U.S. to SI, multiply by	U.S. unit	SI unit	To convert SI to U.S., multiply by
29.5735	fl oz	mL	0.0338
0.0929	ft ²	m ²	10.7639
3.7854	gal	L	0.2642
2.54	inch(es)	cm	0.3937
1	mmho/cm	dS · m ⁻¹	1
28.3495	oz	g	0.0353
28,350	oz	mg	3.5274 × 10 ⁻⁵
7.4892	oz/gal	g · L ⁻¹	0.1335
0.001	ppm	g · L ⁻¹	1000
0.001	ppm	mg · g ⁻¹	1000
1	ppm	mg · L ⁻¹	1
1	ppm	μL · L ⁻¹	1
6.8948	psi	kPa	0.1450
(°F - 32) ÷ 1.8	°F	°C	(1.8 × °C) + 32

After the measurements, leaves were detached, weighed, and kept at -80°C until TSP analysis.

GROWTH ANALYSIS AND FRUIT HARVEST. Stem length and leaf number were measured once a week from 14 Sept. 2008. The rates of stem elongation and leaf development of each plant were calculated as the slopes of the first-order regression equation of time courses of stem length and leaf number, respectively. On 15 Oct. 2008, shoot (leaves and stem, without fruit, above the base of the 10th youngest leaf) was detached, and leaf area and DW of leaves and stem were measured. Leaf area measurement was made with an area meter (LI-3100; LI-COR, Lincoln, NE).

All fruit on the first and second trusses of each plant were harvested at one time on 9 Oct. 2008. The fruit at various growth and developmental stages was classified into six ripeness stages (green, breaker, turning, pink, light red, and red stages) based on the color classification for fresh tomatoes (U.S. Department of Agriculture, 1991). As fruit F1-V rapidly decreases with ripening (Alvarez et al., 2006), only green fruit was used for subsequent growth and biochemical analyses. Fruit between the breaker stage (more than 90% fruit surface in green) and red stage (no longer green) was only used for calculating fruit growth index (FGI) of a green fruit as described below. The fresh weight (FW) and the longest diameter on the equatorial plane of the green fruit were measured. The fruit was then divided longitudinally into three portions through the stem scar and FW of each portion was measured. The three portions were subjected to determination of DW and TSP and F1-V concentrations. Fruit DW was determined after oven-dried at 80°C for at least 5 d, and dry matter percentage (DW/FW) was used for calculating DW per whole fruit. Another portion for TSP determination was kept at -80°C until analysis. The portion for F1-V determination was kept at -80°C , freeze-dried for at least 72 h, pulverized to powder, vacuum-sealed, and stored until analysis.

We defined FGI as an indicator of fruit growth for a green fruit, which is the relative fruit diameter of the fruit to the maximum diameter estimated as the mean diameter of fruit between the breaker and red stage (Matsuda et al., 2010).

BIOCHEMICAL ASSAYS. TSP was extracted from leaves and fruit as described in Matsuda et al. (2009), and its concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard. Fruit F1-V concentration was determined by enzyme-linked immunosorbent assay using a rabbit polyclonal anti-F1-V antibody as described in Alvarez et al. (2006).

DATA ANALYSIS. Significant differences between treatments were tested by *t* test at $P < 0.05$ using statistical software (JMP 9.0.3; SAS Institute, Cary, NC). First- or third-order regression was applied to the relationship between fruit characteristics and FGI where it was significant ($P < 0.05$). Significant differences between treatments in slopes and intercepts in first-order regression equations were tested by analysis of covariance [ANCOVA (Sokal and Rohlf, 1995)] at $P < 0.05$ using the same software.

Results and discussion

EC did not significantly affect the plant growth and development in the present experiment. There was no significant difference between control

and high EC in shoot DW, shoot dry matter, total leaf area, stem elongation rate, or leaf development rate (Table 1). Also, P_n , transpiration rate, and g_s per unit leaf area and leaf TSP concentration per unit DW were not significantly different between treatments (Table 2). These results indicate that the EC level in the high EC treatment ($5.4\text{ dS}\cdot\text{m}^{-1}$) was relatively mild and not as high as that negatively affecting growth and development of whole plant and the leaf characteristics. Similar results were reported by Wu et al. (2004) and Wu and Kubota (2008a).

Because we previously found that fruit TSP and F1-V concentrations per unit DW markedly decreased with fruit growth during the green stage in this transgenic tomato (Matsuda et al., 2010), fruit TSP and F1-V concentrations were compared at various growth stages defined by the fruit size. We defined FGI as an indicator of fruit growth for a green fruit (Matsuda et al., 2010). Similar diameter-based evaluation of the physiological change in developing tomato fruit was also reported by Eltayeb and Roddick (1984). They showed almost the same

Table 1. Mean shoot dry weight, shoot dry matter, and total leaf area at the end of experiment and rates of stem elongation and leaf development of transgenic tomato grown at conventional (control) or high electrical conductivity (EC); $n = 3-6$.

Treatment ^a	Shoot dry wt (g/plant) ^y	Shoot dry matter (%)	Total leaf area (m ² /plant) ^y	Stem elongation rate (cm·d ⁻¹) ^y	Leaf development rate (no./d) ^y
Control	16.8	13.0	0.129	2.23	0.47
High EC	14.8	12.5	0.130	2.24	0.51
<i>t</i> test	NS	NS	NS	NS	NS

^aControl = $2.7\text{ dS}\cdot\text{m}^{-1}$, high EC = $5.4\text{ dS}\cdot\text{m}^{-1}$; $1\text{ dS}\cdot\text{m}^{-1} = 1\text{ mmho}/\text{cm}$.

^y $1\text{ g} = 0.0353\text{ oz}$, $1\text{ m}^2 = 10.7639\text{ ft}^2$, $1\text{ cm} = 0.3937\text{ inch}$.

NS = NS at $P < 0.05$.

Table 2. Mean net photosynthetic rate (P_n), transpiration rate, stomatal conductance (g_s) per unit leaf area, and total soluble protein (TSP) concentration per unit dry weight of young, fully expanded leaves of transgenic tomato grown at conventional (control) or high electrical conductivity (EC); $n = 3-6$.

Treatment ^a	P_n ($\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) ^y	Transpiration rate ($\text{mmol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) ^y	g_s ($\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) ^y	Leaf TSP concn ($\text{mg}\cdot\text{g}^{-1}\text{ dry wt}$) ^x
Control	9.08	5.62	0.280	105
High EC	9.01	5.72	0.290	114
<i>t</i> test	NS	NS	NS	NS

^aControl = $2.7\text{ dS}\cdot\text{m}^{-1}$, high EC = $5.4\text{ dS}\cdot\text{m}^{-1}$; $1\text{ dS}\cdot\text{m}^{-1} = 1\text{ mmho}/\text{cm}$.

^yMeasurements were made under saturating light conditions at a photosynthetic photon flux of $1500\text{ }\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, an atmospheric carbon dioxide concentration of 340 ppm ($\mu\text{L}\cdot\text{L}^{-1}$), a leaf temperature of 28°C (82.4°F) and a leaf-to-air vapor pressure deficit of 1.2 kPa (0.174 psi).

^x $1\text{ mg}\cdot\text{g}^{-1} = 1000\text{ ppm}$.

NS = NS at $P < 0.05$.

Fig. 1. The relationship between fruit (A) fresh weight, (B) dry weight, or (C) dry matter and fruit growth index [FGI (relative fruit diameter to the estimated maximum)] in green fruit of the transgenic tomato grown in control treatment [$2.7 \text{ dS} \cdot \text{m}^{-1}$ (open circle)] or high electrical conductivity (EC) [$5.4 \text{ dS} \cdot \text{m}^{-1}$ (closed triangle)]. Actual fruit diameter at a FGI of 1 was 4.5 cm for control and 4.2 cm for high EC. Data for control were presented in Matsuda et al. (2010). (A) $y = -44.9x^3 + 139x^2 - 72.2x + 11.4$, $r^2 = 0.983$, $P < 0.05$ [control (dashed line)]; $y = 24.7x^3 + 3.89x^2 - 0.281x + 0.193$, $r^2 = 0.953$, $P < 0.05$ [high EC (solid line)]. (B) $y = -1.32x^3 + 5.84x^2 - 2.65x + 0.432$, $r^2 = 0.960$, $P < 0.05$ [control and high EC (dotted line)]. (C) $y = -4.30x + 11.1$, $r^2 = 0.495$ (control); $y = -3.19x + 11.1$, $r^2 = 0.760$, $P < 0.05$ (high EC); $1 \text{ dS} \cdot \text{m}^{-1} = 1 \text{ mmho/cm}$, $1 \text{ cm} = 0.3937 \text{ inch}$, $1 \text{ g} = 0.0353 \text{ oz}$.

pattern of the change in fruit alkaloid content irrespective of its expression based on fruit diameter or the days after anthesis, indicating that diameter-

Fig. 2. The relationship between fruit (A) total soluble protein (TSP) concentration per unit dry weight (DW) or (B) TSP content per fruit and fruit growth index [FGI (relative fruit diameter to the estimated maximum)] in green fruit of the transgenic tomato grown in control treatment [$2.7 \text{ dS} \cdot \text{m}^{-1}$ (open circle)] or high electrical conductivity (EC) [$5.4 \text{ dS} \cdot \text{m}^{-1}$ (closed triangle)]. Actual fruit diameter at a FGI of 1 was 4.5 cm for control and 4.2 cm for high EC. Data for control were presented in Matsuda et al. (2010). (A) $y = -41.2x + 51.7$, $r^2 = 0.755$, $P < 0.05$ [control (dashed line)]; $y = -29.5x + 36.5$, $r^2 = 0.608$, $P < 0.05$ [high EC (solid line)]. (B) $y = 36.1x - 5.0$, $r^2 = 0.677$, $P < 0.05$ (control); $y = 21.2x - 2.4$, $r^2 = 0.393$, $P < 0.05$ (high EC). The result of analysis of covariance is shown in each panel. NS: not significant ($P \geq 0.05$); $1 \text{ dS} \cdot \text{m}^{-1} = 1 \text{ mmho/cm}$, $1 \text{ cm} = 0.3937 \text{ inch}$, $1 \text{ mg} \cdot \text{g}^{-1} = 1000 \text{ ppm}$, $1 \text{ mg} = 3.5274 \times 10^{-5} \text{ oz}$.

based evaluation of fruit physiological dynamics is almost equivalent to time-based evaluation. The maximum diameter for high EC was (mean $\pm$ SE) $4.2 \pm 0.1 \text{ cm}$ and slightly smaller than that for control, $4.5 \pm 0.1 \text{ cm}$.

Green fruit FW at a given FGI was slightly lower in high EC than in control at a given FGI (Fig. 1A), while green fruit for both control and high EC showed a similar increase in DW when plotted against FGI (Fig. 1B). This was because green fruit dry

Fig. 3. The relationship between fruit FI-V (candidate plague vaccine protein) concentration per unit dry weight (DW) and fruit growth index [FGI (relative fruit diameter to the estimated maximum)] in green fruit of the transgenic tomato grown in control [$2.7 \text{ dS} \cdot \text{m}^{-1}$ (open circle)] or high electrical conductivity (EC) [$5.4 \text{ dS} \cdot \text{m}^{-1}$ (closed triangle)] treatment. Actual fruit diameter at a FGI of 1 was 4.5 cm for control and 4.2 cm for high EC. Data for control were presented in Matsuda et al. (2010). $y = -2.94x + 4.12$, $r^2 = 0.428$, $P < 0.05$ [control (dashed line)]. No significant correlation in high EC. $1 \text{ dS} \cdot \text{m}^{-1} = 1 \text{ mmho/cm}$, $1 \text{ cm} = 0.3937 \text{ inch}$, $1 \text{ mg} \cdot \text{g}^{-1} = 1000 \text{ ppm}$.

matter percentage was higher in high EC (Fig. 1C). Such an increase in fruit dry matter percentage (or a decrease in fruit water content) under high EC conditions has been frequently observed (Adams, 1991; Adams and Ho, 1989). There also was a slight difference in red fruit FW between high EC [(mean $\pm$ SE) $30.5 \pm 1.8 \text{ g}$] and control ($36.8 \pm 3.5 \text{ g}$), although it was not statistically significant, suggesting that the decrease in fruit water content in high EC was not so large as to have led to a significant FW-based yield decrease. We did not observe any physiological disorders on fruit such as blossom-end rot.

Fruit TSP concentration per unit DW decreased as FGI increased in both control and high EC (Fig. 2A). On the other hand, TSP content per whole fruit linearly increased with increasing FGI (Fig. 2B), indicating that dry matter accumulation had a greater impact on TSP content than the decrease in TSP concentration. In either relationship, the intercept of the first-order regression equation was significantly smaller in high EC than in control, while there was

Table 3. Mean F1-V (candidate plague vaccine protein) concentration per unit dry weight and F1-V content per fruit for green fruit (all growth stages) of transgenic tomato grown at conventional (control) or high electrical conductivity (EC); $n = 18$ (control), $n = 10$ (high EC).

Treatment ^a	F1-V concn (mg·g ⁻¹ dry wt) ^b	F1-V content (mg/fruit) ^b
Control	2.02	1.37
High EC	1.24	0.77
<i>t</i> test	*	*

^aControl = 2.7 dS·m⁻¹, high EC = 5.4 dS·m⁻¹; 1 dS·m⁻¹ = 1mmho/cm.

^b1 mg·g⁻¹ = 1000 ppm, 1 mg = 3.5274 × 10⁻⁵ oz.

*indicates significant at $P < 0.05$.

no significant difference in slope by ANCOVA. This means that, at a given FGI, both TSP concentration and content in high EC were significantly lower than those in control. These results indicate that the high EC treatment decreased overall soluble proteins at a given FGI.

Figure 3 shows the relationship between F1-V concentration per unit DW and FGI. F1-V concentration decreased linearly as fruit grew in control. Although it also tended to decrease with increasing FGI in high EC treatment, no statistically clear relationship was observed. The reason why F1-V concentration linearly decreased in control but not in high EC is unclear. There was no apparent correlation between F1-V content per whole fruit and FGI for both control and high EC treatment (data not shown). As ANCOVA could not be applied to F1-V concentration per unit DW and F1-V content per whole fruit for comparison of control and high EC, averages over entire green stage were calculated (Table 3). Both F1-V concentration and content were significantly higher in control than in high EC. Thus, our results indicated that the high EC treatment decreased overall soluble proteins including F1-V compared with the control.

To summarize the results, the high EC treatment applied here did not alter plant growth, development, leaf gas exchange, or fruit dry matter accumulation, but did specifically lower fruit TSP concentration including F1-V concentration per unit DW and their contents per whole fruit in the transgenic tomato. Generally, in salt- and water-stressed parts of a plant, the protein content can decrease owing to the decreased rate of protein synthesis, the increased rate of

proteolysis, or both (Dubey, 1999). This could be at least partly responsible for the lower TSP and F1-V concentration observed in the high EC treatment. Our results suggest that adjustment of nutrient solution EC at an appropriate level is necessary for preventing salinity stress to the plant and a subsequent decrease in fruit soluble proteins including F1-V. This reduction in vaccine protein yields occurred with a moderate salinity stress, with no apparent negative effect on growth and development, requiring us to manage nutrient supply carefully. Although moderate water stress was reportedly effective in enhancing the amount of subunit vaccine candidate against anthrax in leaves of transgenic tobacco (Stevens et al., 2007), moderate salinity stress was not effective or even had a negative influence in our transgenic tomato fruit.

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