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Expression Analysis of *LeNHX1* Gene in Mycorrhizal Tomato under Salt Stress

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The plant growth, stem sap flow, Na^+ and Cl^- content, and the expression of vacuolar Na^+/H^+ antiporter gene (*LeNHX1*) in the leaves and roots of tomato under different NaCl stresses (0.5% and 1%) were studied to analyze the effect of arbuscular mycorrhizal fungi (AMF) on Na^+ and Cl^- accumulation and ion exchange. The results showed that arbuscular mycorrhizal (AM) plant growth and stem sap flow increased and salt tolerance improved, whereas Na^+ and Cl^- accumulated. Na^+ significantly decreased, and no significant decline was detected in Cl^- content after AMF inoculation compared with the non-AM plants. The *LeNHX1* gene expression was induced in the AM and non-AM plants by NaCl stress. However, AMF did not improve the *LeNHX1* level, and low expression was observed in the AM tomato. Hence, the mechanism that reduced the Na^+ damage to tomato induced by AMF has little relation to *LeNHX1*, which can export Na^+ from the cytosol to the vacuole across the tonoplast.

Keywords: arbuscular mycorrhizal fungi, salt stress, Na^+ and Cl^- accumulation, Na^+/H^+ antiporter

Introduction

Almost all land plants have developed a symbiosis with arbuscular mycorrhizal fungi (AMF). Under diverse stress conditions, most higher plants are colonized by AMF, which can have manifold beneficial effects on plant growth (Trimble and Knowles, 1995; Al-Karaki, 2000; Kaya *et al.*, 2003). Salt stress and water deficit are the most common stresses that affect crops in arid and semi-arid regions. The use of new biological methodologies is a necessary and practical way to improve agricultural plant tolerance to salinity. Tomato is popularly cultivated, and its production is often limited by salinity. In recent years, studies have indicated that AMF can increase plant growth and uptake of nutrients, reduce yield losses of tomato under saline conditions, and improve salt tolerance of tomato (Al-Karaki, 2000; Abdel Latef and Chaoxing, 2011). Root colonization by AMF involves a series of morpho-physiological and biochemical events regulated by the interaction of plant and fungus, as well as by environmental factors (Blumwald *et al.*, 2000). The physiological and biochemical mechanisms that improve the salt tolerance of AM tomato are still unclear and have not yet been elucidated at a molecular level.

Plant salt tolerance itself is a complex trait (Shi *et al.*, 2000) to which many different factors may contribute, such as generation of osmoprotectants in the cytoplasm, energy supply by ATPase for the export of Na^+ and Cl^-, specific transport proteins for the transfer of these ions into the vacuole or into the apoplastic spaces, additional water supply mediated by aquaporins to maintain osmobalance, and so on (Hasegawa *et al.*, 2000). When plants are stressed by salinity, the toxic effects of the ions are mainly Na^+ and Cl^- (Zhu, 2003). Na^+ can be exported either into the apoplast by a plasma membrane-associated Na^+/H^+ antiporter or into vacuoles by a tonoplast-associated Na^+/H^+ antiporter (DuPont, 1992; Blumwald *et al.*, 2000). The latter is especially important for leaf cells that have a limited capacity for apoplastic Na^+ discharge (Munns, 2002). It has been shown that an overexpression of a tonoplast-associated Na^+/H^+ antiporter in tomato can significantly increase salt tolerance of the transgenic plants (Zhang and Blumwald, 2001). A similar achievement was also reported for *Arabidopsis* (Apse *et al.*, 1999) or rice (Fukuda *et al.*, 1999; Ohta *et al.*, 2002). In tomato, there are at least 4 *LeNHX* type genes (*LeNHX1~4*) (Rodríguez-Rosales *et al.*, 2009). *LeNHX1*, *3* and *4* are tonoplast Na^+/H^+ antiporter (Pardo *et al.*, 2006) and *LeNHX2* has been shown to be a K^+/H^+ transporter (Venema *et al.*, 2003). Recently, Gálvez *et al.* (2012) had studied expression of 4 tomato *NHX* (*LeNHX 1~4*) in wild and cultivated species by RT-PCR analysis. It shows that the wild species is more tolerant and *NHX* expression is induced more, as well as accumulates more Na^+ in aerial parts of the plant. *LeNHX1* expression is obviously induced especially by the short treatment.

Overexpression of Na^+/H^+ antiporters in plants or microorganisms renders them more tolerant to salt stress (Zhang *et al.*, 2001; Waditee *et al.*, 2002). Rabie and Almadini (2005) suggested that AMF protect leaf metabolism from Na^+ toxicity. Thus, analyzing expression of Na^+/H^+ antiporter gene in dependence on salt and mycorrhizal colonization is of particular interest in the present study. So far, the effect of AMF on salt-stressed plants on the molecular level is poorly understood. Thus, this paper focuses on the accumulation of Na^+ and Cl^- and the expression of the vacuolar Na^+/H^+ antiporter gene (*LeNHX1*) in mycorrhizal tomato based on analysis of salt resistance under NaCl stress.

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Materials and Methods

Plant material and experimental conditions

Seeds of tomato (*Lycopersicum esculenyum* cv. Zhongzha No. 9) were obtained from the Institute of Vegetables and Flowers, CAAS, Beijing, P. R. China. The seeds were sterilized by immersion in 70% alcohol for 5 min. Then, they were rinsed four times with distilled water and stored to germinate on wet filter paper in Petri dishes at 28°C. After three days, the seeds were planted in polystyrene trays. At the same time, half of the pots (AM plants) were inoculated with 10 g *Glomus mosseae* (provided by the Hungarian Institute of Soil Research) per pot. The non-AMF plants received the same weight of autoclaved inoculums. The inoculums were placed adjacent to each seeding root. The 30-day-old seedlings with uniform sizes were transplant in 13×13 cm plastic pots containing 0.88 kg organized soil mixture (organic manure, soil, and straw were in the proportion of 1:2:1). The soil mix was collected from the greenhouse of the Institute of Vegetables and Flowers, and then sterilized (160°C, 4 h). The soil properties were as follows: pH=7.26, 11.1% organic matter, 0.15% available phosphorus, 451 mg/kg available nitrogen, and 518 mg/kg available potassium. The experimental pots were placed in a greenhouse with natural light at 28°C/20°C (day/night) from September to December. The photon flux density ranged from 600 μmol/m^2/s^2 to 1200 μmol/m^2/s^2, and the relative humidity was between 65% and 95%.

Mycorrhizal fungus inoculums

Mycorrhizal fungus inoculums, consisting of spores, soil, hyphae, and infected clove (*Trifoliumrepens*) root fragment from a stock culture of *G. mosseae*, was provided by the Hungarian Institute of Soil Research. The inoculated dosage was 10 g inoculums per pot, containing 720 spores.

Experimental design

The experimental design consisted of six treatments, crossing two mycorrhizal inoculation levels (non-AMF and *G. mosseae*), with three soil salt levels (treated with 0%, 0.5%, and 1% NaCl solution). The pots were arranged in a completely randomized block design. Six replicates of each treatment were performed, totaling 36 pots (2 seedlings/pot).

From the 45th day after AMF inoculation, the plants (salt treatments) were irrigated every 2 or 3 days with 0.5% and 1% NaCl-water solution. The salt-free treatments were irrigated with tap water (EC=0.8 mS/cm). The soil EC values reached 0.9, 4.2, and 7.1 mS/cm in the 0%, 0.5%, and 1% treatments, respectively. Then, the salt solution irrigation was stopped. The dry weight, leaf area, Na^+, Cl^-, and stem sap flow were measured and the electrical conductivity (EC) in the growth substrate was kept constant until 40 d after salt stress. EC was regularly monitored using a Model LF539 Conductivity Meter (WTW, Germany). When leaching occurred, the leachate was collected and then added back to the soil to maintain the salinity treatments near the target levels.

Table 1. Effect of salinity on root colonization

NaCl (%)	AMF status	Root colonization (%)	
		0 d after salt stress	40 d after salt stress
0	AM	58.6 aA	60.8 aA
	NAM	0.0 bB	0.0 dD
0.5	AM	58.4 aA	44.9 bB
	NAM	0.0 bB	0.0 dD
1	AM	60.2 aA	32.3 cC
	NAM	0.0 bB	0.0 dD

Note: Date were analyzed by Duncan's multiple new range test and the different capital and little letters indicate significant differences at $P<0.01$ and $P<0.05$ level respectively. AM, tomato inoculated with AMF; NAM, tomato without AMF.

Measured parameters

Biomass measurements: The shoot and the root were separated 40 d after salt stress. The dry weight (DW) was measured after oven drying at 80°C for 2 d.

Leaf area measurements: The leaf area of the fourth leaf from the bottom of the control and the treated plants was measured using a Portable Leaf Area meter AM300 (England) every 5 d during the salt stress period.

Stem sap flow analysis: At 5 d after salt stress, the stem sap flows of the AM and non-AM plants under 1% NaCl stress were measured using the plant growth monitoring system LPS-05MD (Israel). The probe was fixed in the plant stem for about 24 h. The stem sap flow for four consecutive days was also observed.

Sodium and chloride analyses: The sodium concentration was measured using a flame emission spectrometer after digestion in flasks with H_2SO_4-salicylic acid-H_2O_2 (Chen *et al.*, 2001). Chloride determination was carried out using the method of Chen and co-workers (2001).

Isolation of RNA and synthesis of cDNA: At 30 d after salt

Table 2. Effect of AMF on dry weight and leaf area of tomato under NaCl stress

NaCl (%)	AMF status	Leaf area (cm^2)					Dry weight (g)	
		Days after salt stress (d)				Increasement (cm^2)	Shoot	Root
		0 d	5 d	10 d	15 d	0 d–15 d	40 d	
0	AM	10.12±0.12a	13.47±0.12a	15.25±0.15a	19.76±0.13a	9.64a	5.3±0.13a	0.95±0.02a
	NAM	9.37±0.11b	12.1±0.08b	13.32±0.16b	17.49±0.17b	8.12b	4.5±0.11b	0.74±0.05b
0.5	AM	9.96±0.15a	11.95±0.15b	13.06±0.12b	14.30±0.13c	4.34c	3.35±0.1c	0.62±0.03c
	NAM	9.14±0.09b	10.96±0.12cd	11.79±0.14c	12.33±0.15d	3.19d	2.8±0.11d	0.45±0.02d
1	AM	10.23±0.11a	11.55±0.13bc	11.98±0.11c	12.28±0.21d	2.05de	2.35±0.09e	0.29±0.01e
	NAM	9.45±0.14b	10.56±0.13d	11.17±0.09d	11.37±0.11e	1.92e	1.91±0.06f	0.18±0.01f

Note, Data were analyzed by Duncan's multiple new range tests. The small letters indicate significant differences at $P<0.05$.
AM, tomato inoculated with AMF; NAM, tomato without AMF.

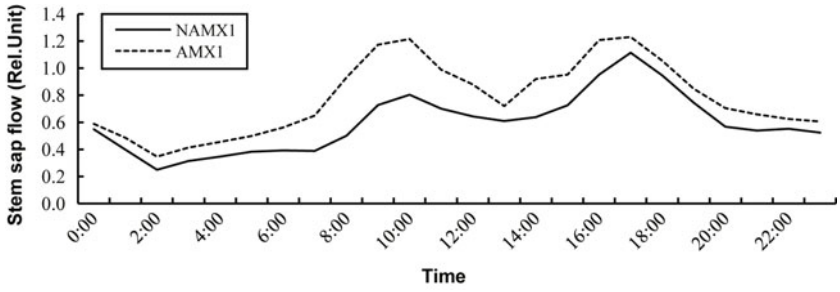

Fig. 1. Effect of AMF on stem sap flow in tomato during one whole day of NaCl stress. NAMX1, non-AM plant under 1% NaCl stress; AMX1, AM plant under 1% NaCl stress.

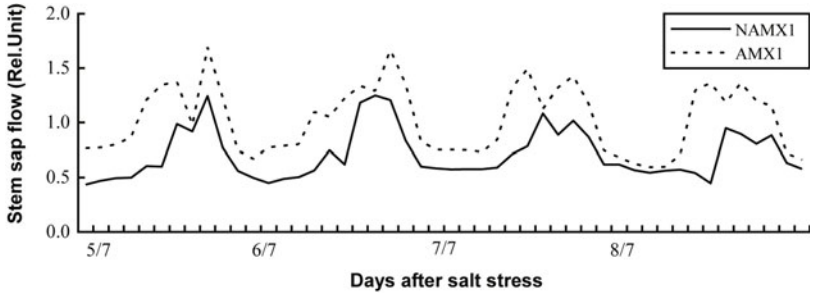

Fig. 2. Effect of AMF on stem sap flow in tomato under NaCl stress on consecutive days. NAMX1, non-AM plant under 1% NaCl stress; AMX1, AM plant under 1% NaCl stress.

stress, total RNA from the roots and leaves of the AM and non-AM plants was isolated using the TRIpure Reagent Kit. The Superscript Preamplification System for First Strand Synthesis (Invitrogen) was used for the synthesis of cDNA from the total RNA of the tomato roots or leaves.

Quantification of mRNA through real-time PCR: In the case of the Na^+/H^+ antiporter genes, the procedure was performed on the vacuolar Na^+/H^+ antiporter gene (*LeNHX1*) in a GeneAmp7000 Sequence Detector System (Applied Biosystems, ABI) with the following thermal program: 2 min at 95°C, 30 sec at 94°C, and 1 min at 55°C. The fluorescence of the amplificates was detected using the SYBR Green PCR Master Mix (Applied Biosystems), and then quantified using the GenAmp7000 sequence detector system software. The primers for the real-time PCR were *LeNHX1* F: ATGTTGT TGGTGCCGTCG; *LeNHX1* R: AGGCTGCTCGTCCTGATT. The amplified length was 183 bp, and the gene sequence was AJ306630.

Real-time RT-PCR was performed on ABI PRISM 7000 Sequence Detection System using the SYBR Green PCR.

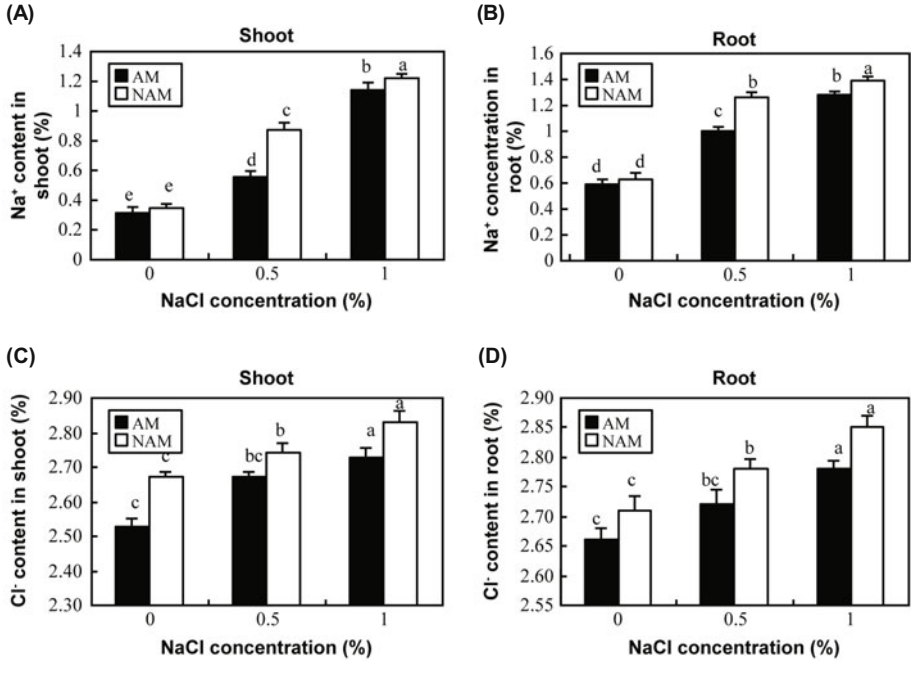

Fig. 3. Effect of AMF on Na^+ (A), (B) and Cl^- (C), (D) contents in shoots and roots of tomato under NaCl stress.

Results

Characterization of tomato plants after AMF colonization under salt stress

Effect of salinity on root colonization: According to Table 1, salt concentration significantly changed the root colonization. With the salt concentration increasing, the root colonization reduced. It was also observed that the root colonization reduced after a long salt stress time.

Effect of AMF on DW and leaf area of tomato under NaCl stress: NaCl stress significantly reduced plant growth, and the growth improved after AMF inoculation (Table 2). Higher leaf area was detected in the AM plants. At 40 d, the DW of the AM seeding were 19.2%, 22.1%, and 26.3% higher than those of the non-AM controls under 0%, 0.5%, and 1% NaCl stress, respectively. The effect of AMF on the seedlings was more obvious under higher salt stress.

Effect of AMF on stem sap flow in tomato under NaCl stress: Stem sap flow and plant transpiration are closely related (Nagler *et al.*, 2003). At 5 d after salt stress (1% NaCl), similar stem sap flow diurnal variations were observed in the AM and non-AM plants. However, the AM plant always maintained a higher value compared with that of the non-AM plant (Fig. 1). This behavior shows that the AM plant has strong transpiration and root water uptake capacities, which imply strong growth potential and salt tolerance. With continuous salt stress, the maximum value gradually decreased, but the AM plant still maintained higher levels (Fig. 2).

Accumulation of Na^+ and Cl^-

The Na^+ and Cl^- contents in the shoots and roots increased as the salinity increased (Fig. 3), and the AM plants had lower contents than the non-AM plants. The Na^+ contents in the shoots and roots of the non-AM seedlings were 1.57 and 1.26 times more than those of the AM plants under 0.5% salt stress, and 1.11 and 1.22 times more under 1% salt stress. AM colonization seemed to reduce Na^+ under 0.5% salt stress more remarkably ($p < 0.05$) (Figs. 3A and 3B). However, the decline in Cl^- content due to AMF colonization was not obvious (Figs. 3C and 3D) ($p < 0.05$).

Expression studies of the vacuolar Na^+/H^+ antiporter gene (*LeNHX1*)

For the expression analyses (Fig. 4), RNA was isolated from the leaves and roots of both the NaCl-stressed and the non-NaCl-treated control plants to assess the expression of *LeNHX1*. In the control, the transcript level in the leaves or roots was similar in both AM and non-AM plants. However, mycorrhizal colonization significantly reduced the transcript amount under salt stress. The AM or non-AM plants showed stronger relative signal intensities than the non-NaCl-treated plants. The non-AM plants showed higher intensity than the AM plants.

Discussion

Saline soils usually contain a variety of sodium salts, such as NaCl and Na_2SO_4. Excessive accumulation of Na^+ and Cl^- can cause ion toxicity in plants. At the same time, the absorption of other ions is also affected. Thus, the cells and some macromolecules are damaged, and salt stress causes physiological and biochemical metabolic disorders in plants (Zhu, 2001).

In the transpiration process, the water absorbed from the soil by the crop roots is sent through the stalk to the leaves and then emitted to the atmosphere through the leaf stomata. In the process, the liquid in the stem is in a flow state. Plant root is damaged under salt stress, and root water uptake is blocked, which affect plant stem sap flow (Nagler *et al.*, 2003). In our experiment, NaCl stress significantly reduced stem sap flow and growth of tomato, and both improved after AMF inoculation. Noticeably, AMF improved the salt tolerance, which is consistent with previous studies (Al-Karaki, 2000; Abdel Latef and Chaoxing, 2011).

In this study, with increased salt concentration, the Na^+ and Cl^- in the plants continued to accumulate and the similar achievement was also reported in previous study (Guerrier, 1996). However, the tolerant AM plants accumulated less Na^+ compared to that in the control plants and AMF can mainly reduce Na^+ accumulation in plants in response to salt stress. In general the expression pattern of the NHX isoforms is in accordance with a role for these transporters in Na^+ accumulation (Gálvez *et al.*, 2012). Na^+ can be transported out of the cytoplasm into either the vacuole or the apoplasm by the Na^+/H^+ antiporter protein. This process can reduce ion damage due to Na^+ accumulation (Blumwald *et al.*, 2000). Real-time PCR analysis showed that expression of the *LeNHX1* gene in all the plants was enhanced in response to salt stress (Fig. 4). It was similar with the results of the study on tomato (Gálvez *et al.*, 2012). However, the *LeNHX1* gene in the AM plants was significantly lower than that in the non-AM plants which showed AMF did not improve the *LeNHX1* levels. Hence, the mechanism that reduces Na^+ damage to plants induced by AMF has little relation to *LeNHX1*, which can export Na^+ from the cytosol to the vacuole across the tonoplast. It is somewhat surprising

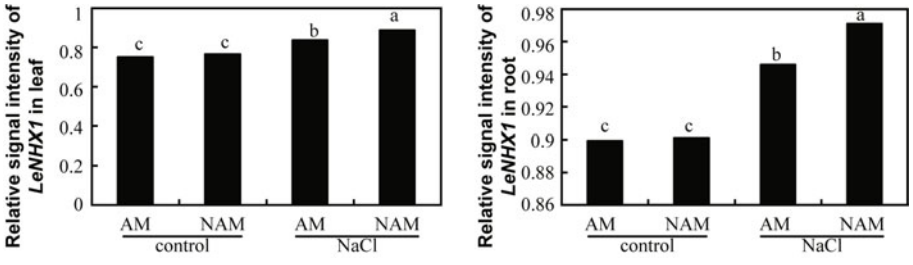

Fig. 4. Real-time PCR analysis of *LeNHX1* in leaves and roots of tomato under salt stress.

to us how the AM plants reduce the Na^+ accumulation in root or shoot? AMF in saline soils can decrease plant yield losses by increasing their salt tolerance (Al-Karaki, 2000; Abdel Latef and Chaoxing, 2011). In addition, AMF has severe impacts on the NaCl allocation within roots (Scheloske *et al*., 2004). Several publications suggest that AMF can increase plant growth or change K^+/ Na^+, P/ Na^+ and thus dilute the Na^+ concentration in the plant relatively (Jarrell and Beverly, 1981; Al-Karaki, 2000). The mechanism of salt tolerance induced by AMF in the molecular level is still unclear. The study of the Na^+/H^+ antiporter is a new highlight in plant salt tolerance, especially in mycorrhizal plants, and it needs further research, with new Na^+/H^+ antiporter genes being found and cloned.

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