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Effect of nano-silicon application on the expression of salt tolerance genes in germinating tomato (*Solanum lycopersicum* L.) seedlings under salt stress

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Abstract

Nowadays, nano-silicon (N-Si) has been used to enhance seed germination, plant growth, and improvement of photosynthetic quantum under environmental stresses. In this study, we examined the effects of different doses of N-Si on the salt tolerance of tomato (*Solanum lycopersicum* L.) plants during germination. The tomato seeds were treated with different doses of N-Si and were germinated under salt stress. The seed germination and seedling growth of tomato plants were significantly inhibited by salt stress. However, this inhibition was alleviated by the exposure to N-Si. The germination percentage and germination rate of tomato seeds and the root length and fresh weight of tomato seedlings were increased after exposure to N-Si under NaCl stress. The expression profiles of salt stress genes were investigated with the semi-quantitative RT-PCR. Of the fourteen investigated salt stress genes, four genes, *AREB*, *TAS14*, *NCED3* and *CRK1*, were upregulated, and six genes, *RBOH1*, *APX2*, *MAPK2*, *ERF5*, *MAPK3* and *DDF2*, were downregulated with exposure to N-Si under salt stress. The gene expression patterns associated with exposure to N-Si suggested a potential involvement of N-Si in the plant's response to stress, an indication that N-Si might be useful to improve plants' tolerance of salinity.

Keywords: *Solanum lycopersicum* L., nano-silicon, seed germination, seedling growth, salinity, stress genes.

Abbreviations: APX2_cytosolic ascorbate peroxidase 2, AREB_abscisic acid responsive element-binding protein, CAC_clathrin adapter complexes, CRK1_cysteine-rich receptor-like protein kinase 42-like, DDF2_dwarf and delayed flowering 2, ERF_ethylene response factor, H_2O_2_hydrogen peroxide, LOX1_linoleate 9S-lipoxygenase B, MAPK_mitogen-activated protein kinase, NaCl_sodium chloride, NCED3_9-cis-epoxycarotenoid dioxygenase, NHX6_NA+/H+ antiporter 6, N-Si_nano-silicon, N-SiO_2_nano-silicon dioxide, P5CS_delta-1-pyrroline-5-carboxylate synthetase, RBOH1_respiratory burst oxidase, RT-PCR_reverse transcription PCR, SGN_Sol Genomics Network, Si_silicon, SOS2_salt overly sensitive 2, TAS14_abscisic acid and environmental stress-inducible protein

Introduction

A menace to agriculture, salinity stress is a major environmental factor that affects crop production. The increased salinization of arable land is expected to have global effects on the production of crops (Pitman and Läuchli, 2002). Therefore, increasing the tolerance of crop plants to salinity stress must assume priority in agricultural research. In crop plants, various genes are responsible for salinity resistance and are involved in the process of salt tolerance. These genes limit the rate of salt uptake from the soil and the transport of salt throughout the plant, and also adjust the ionic and osmotic balance of cells in the plant (Munns, 2002). The first phase of plant growth is a response to the effects of salt outside the plant. The salt in the soil solution reduces leaf growth and, to a lesser extent, root growth (Munns, 2002; Zhao et al., 2010). The molecular control mechanisms for salt stress tolerance are dependent on the regulation of the expression of specific stress genes (Wang et al., 2003).

Silicon (Si) is the second most abundant element in the soil. However, silicon is not considered an essential element. Recently, numerous studies have shown that treatment with silicon significantly alleviated salt, drought, chilling and freezing stress in plants. The Si treatments were considered beneficial to plant growth and production (Liang et al., 2007; Ma and Yamaji, 2008). Silicon nanoparticles (N-Si) have been implicated in crop improvements. Many reports indicate that appropriate concentrations of N-Si increase seed germination (Karunakaran et al., 2013), plant growth (Yuvakkumar et al., 2011) and plant resistance to hydroponic conditions (Suriyaprabha et al., 2012). Recently, the role of N-Si in the mitigation of salt stress received worldwide attention because of reports about the ability of N-Si to counteract the negative effects of salt on plant growth rates. Haghighi et al. (2012) found that N-Si reduced the negative effects of salinity on tomatoes during germination, in which treatment with 1 mM N-Si increased the germination rate, root length and dry weight of tomato plants in 25 mM NaCl conditions. By contrast, 2 mM N-Si inhibited the germination of plants grown in 50 mM NaCl conditions. Sabaghnia and Janmohammadi (2014) found that the application of 1 mM nano-silicon dioxide (N-SiO_2) provided considerable alleviation of the adverse effects of salt stress on the germination percentage of lentil seeds. The length of the roots and shoots, seedling weight, mean germination time, seedling vigour index and seed reserve mobilization were also positively affected. In a study by Abdul Qados and Moftah (2015), the application of Si and N-SiO_2 significantly increased the germination of *Vicia faba* L. seeds under

salinity stress. Among the treatments, the 2 mM Si and the N-SiO_2 treatments improved germination characteristics. Relative water content, plant height, and fresh and dry weights also increased in treatments with Si or N-Si. In basil (*Ocimum basilicum*), leaf dry and fresh weights, chlorophyll content and proline content increased after treatment with N-Si under salinity stress (Kalteh et al., 2014).

In response to stress, plants activate a number of defence mechanisms that function to increase the tolerance to the adverse conditions. Furthermore, a large array of genes is activated by the stress, and several proteins are produced that join the pathways that lead to a synergistic enhancement of stress tolerance (Wang et al., 2003). The genes for salinity tolerance in tomato were identified in many reports and were published in the Sol Genomics Network (SGN) database (SGN, 2015; Aoki et al., 2010). Of these genes, those that code for transcription factors, which regulate many genes in response to stress, include *abscisic acid responsive element-binding protein (AREB)* (Orellana et al., 2010; Bastías et al., 2014), *ethylene response factor 5 (ERF5)* (Pan et al., 2012) and *ERF1* (Huang et al., 2004; Li et al., 2009). Osmolytes are among the proteins produced by genes that are induced under salinity stress and have a primary role in osmotic adjustments at high concentrations of salt. They include *delta-1-pyrroline-5-carboxylate synthetase (P5CS)* (Hong et al., 2000), *abscisic acid* and *environmental stress-inducible protein (TAS14)* (Muñoz-Mayor et al., 2012). The genes responsible for controlling K+ or Na+ uptake from the soil and transport within the plant were also identified in transgenic tomatoes under salinity stress as being responsible for ion channels and transporters, such as the *salt overly sensitive 2 (SOS2)* (Olías et al., 2009; Candar-Çakir et al., 2014) and the *NA+/H+ antiporter 6 (NHX6)* (Zhang and Blumwald, 2001). In plants under salt stress, the antioxidative defence system involves antioxidative enzymes such as *cytosolic ascorbate peroxidase 2* (*APX2*), which is expressed in tomatoes in saline conditions (Zou et al., 2005). The signalling pathways that respond to salt stress in tomato include many signalling molecules, such as *mitogen-activated protein kinase 2 (MAPK2)* and *MAPK3* (Stulemeijer et al., 2007). Some additional genes involved in energy metabolism are also expressed in tomatoes under salt stress, such as *respiratory burst oxidase (RBOH1)*, which is an NADPH oxidase (Zhou et al., 2014).

Research in biotechnology seeks to reveal the molecular bases of these defence mechanisms with the goal to implement nanoparticles for the improvement of plant tolerances to the environmental stresses. The aim of this study was to reveal the molecular mechanisms that mediated salinity tolerance in tomato plants with the achievement of two objectives: to determine the effect of the N-Si dosage on the plant's resistance to salt stress with measurements of germination efficiency and seedling growth; and to investigate the molecular mechanisms responsible for the positive effects of N-Si on plants' resistance to salinity with the expression profiles of the tomato salt tolerance genes using the semi-quantitative reverse transcription PCR (semi-quantitative RT-PCR) technique.

Results and Discussion

The effect of N-Si on seed germination and seedling growth of tomatoes under salt stress

In the current research, we studied the role of N-Si in enhancing tolerance of tomatoes to salinity and highlighted the molecular mechanisms that mediated salinity tolerance in tomato plants with an investigation of the expression profiles of salt tolerance genes at germination stage. Three control groups were used in germination experiments. For the first control, the plants were grown in 150 mM NaCl without exposure to N-Si (NaCl150), which was the control for the N-Si treatments with 150 mM NaCl. For the second control, the plants were grown in 200 mM NaCl without exposure to N-Si (NaCl200), which was the control for the N-Si treatments with 200 mM NaCl. For the third control, the plants were grown in distilled water only (non-treated control), which was the control for the N-Si treatments without and with NaCl treatments and for the NaCl treatment without N-Si. Our results indicated that N-Si played an important role in moderating the inhibition of seed germination and plant growth in saline environments with the induction of salt tolerance in tomato plants. With exposure to N-Si, the germination percentage, germination rate, root length and seedling fresh weight of tomato plants all increased under NaCl stress.

Table 1. shows that germination percentage, germination rate and mean germination time for the NaCl treatments (150 and 200 mM) with the different N-Si concentration treatments were significantly different from those of the non-treated plants. These parameters were not affected by the treatment with N-Si only without the NaCl stress, except for mean germination time, which increased at 3 mM of N-Si compared with the non-treated control (Fig 1). The mean germination time also improved under salt stress with N-Si treatment. Previous reports found that N-Si had positive effects on seed germination, seedling growth and yield, as well as the physiology and metabolism in different plant species (Siddiqui et al., 2015). These findings suggested that N-Si might be involved directly or indirectly in both the morphological changes and the physiological processes in plants. Thus, our findings were consistent with previous findings that treatment with N-Si had a protective role in the germination of tomato seeds under salt stress.

The root lengths and fresh weights of tomato seedlings were significantly reduced under the NaCl stress and N-Si treatments compared with the non-treated plants, whereas the seedling dry weights were not affected after N-Si treatments compared with the non-treated control (Table 2). These parameters were not affected by treatment with N-Si alone without NaCl stress, with the exception of fresh weight, which decreased at 0.5 and 1 mM of N-Si compared with non-treated controls. The longest root length value for tomato plants was observed at 3 mM of N-Si in the NaCl150 stress treatment, the lowest mean germination time was recorded at 1 mM of N-Si under the NaCl150 stress treatment, and the highest germination rates and fresh weight values were at 2 mM of N-Si under the NaCl150 stress treatment (Fig 1). The exposure to 1 mM of N-Si was sufficient to enhance the tomato mean germination time and increase the root length under the NaCl200 salinity level, and the highest germination rates and fresh weight values were recorded at 3 mM of N-Si under the NaCl200 stress treatment. Similarly, treatment with N-Si alleviated the inhibitory effects of salt stress and increased seed germination and growth characteristics of *Cucurbita pepo* L. with a reduction in the levels of malondialdehyde and hydrogen peroxide (H_2O_2) and in electrolyte leakage. The application of N-Si increased the rate of photosynthesis, mesophyll conductance, and plant water use efficiency of tomato plants under saline stress (Haghighi and Pessarakli, 2013). In previous reports, the identical alleviation of the adverse effects of salt stress was also observed with N-SiO_2 treatment. The application of N-SiO_2 reduced the degradation of chlorophyll and increased net photosynthetic rate, stomatal conductance, transpiration rate, and water use efficiency (Siddiqui et al., 2014). The application of N-SiO_2 also significantly increased seed

Table 1. Influence of different concentrations of N-Si on tomato germination under two levels of salinity.

NaCl mM		Control	N-Si mM 0.5	1	2	3
0	GP%	100.00± 1.00	100.00± 3.00	100.00± 2.00	100.00± 3.00	100.00± 3.00
	GR (seeds/day)	14.807± 0.172	14.693± 0.269	14.887± 0.098	14.803± 0.211	14.943± 0.098
	MGT (day)	31.790± 0.106	31.743± 0.223	31.843± 0.103	31.900± 0.118	31.977*± 0.040
150	GP%	97.78♦± 2.00	100. 00*± 2.00	100. 00*± 1.92	98.89± 1.92	100. 00*± 4.00
	GR (seeds/day)	12.947♦♦± 0.632	13.117♦± 0.789	11.973♦♦± 0.871	14.067*♦± 0.337	12.857♦♦± 0.420
	MGT (day)	31.177± 0.500	29.580*♦♦± 0.383	28.81**♦± 1.55	30.877♦± 0.569	30.247♦♦± 0.400
200	GP%	94.45♦♦± 6.94	98.89*± 1.92	97.78*± 3.85	97.78*± 1.92	98.89*± 1.92
	GR (seeds/day)	10.37♦♦± 1.15	11.037♦♦± 0.309	10.55♦♦± 0.137	10.853♦♦± 0.743	11.233*♦♦± 0.159
	MGT (day)	29.62♦± 1.60	28.613♦♦± 0.298	27.857*♦♦± 0.280	28.237*♦♦± 0.321	29.043♦♦± 0.993

Values are the M (mean) ± SD (standard deviation) of three replications. (*) indicates significant effects at a 0.05 probability level, and (**) indicates significant effects at a 0.01 probability level, related to the NaCl150 or NaCl200 salinity treatments. (♦) indicates significant effects at a 0.05 probability level, and (♦♦) indicates significant effects at a 0.01 probability level, related to the non-treated control. GP = germination percentage, GR = germination rate, MGT = mean germination time, % = percent and mM = millimolar

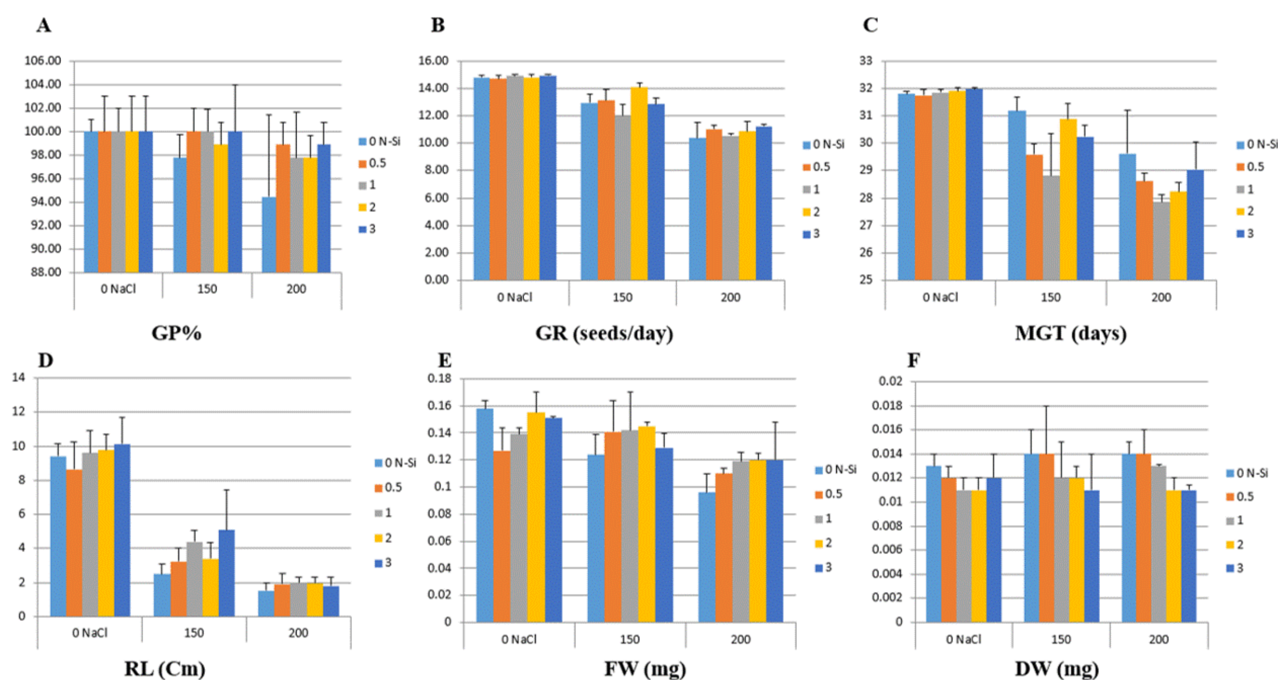

Fig 1. Influence of the different concentrations of N-Si on germination efficiency and seedling growth of tomato plants under salt stress. Horizontal axis in all charts present NaCl concentration (0, 150 and 200 mM) as salt stress. Vertical axis presents: **A:** germination percentage (GP), **B:** germination rate (GR), **C:** mean germination time (MGT), **D:** root length (RL), **E:** seedling fresh weight (FW) and **F:** seedling dry weight (DW) of tomato. Columns with different colours present different N-Si concentrations (0, 0.5, 1, 2 and 3 mM). Different letters indicate a significant difference among treatments at the 0.01 and 0.05 levels by t-test.

germination, plant height, fresh and dry weights and total yield of *Vicia faba* L. Seed quality, as indicated by nutrient content, was also improved with the application of N-Si (Abdul Qados and Moftah, 2015). The treatment with N-SiO_2 also increased seed germination percentage, improved mean germination time, and increased the seed germination index, seed vigour index, and seedling fresh and dry weights of tomato (Siddiqui and Al-Whaibi, 2014) and lentil (Sabaghnia and Janmohammadi, 2014; Sabaghnia and Janmohammadi, 2015) under saline stress.

Expression pattern of tomato salt stress genes in response to N-Si under salt stress

The expression of the salt stress genes was investigated with semi-quantitative RT-PCR using the cDNA isolated from tomato seedling tissues.

Table 2. Influence of concentrations of N-Si on tomato seedling growth under two levels of salinity.

NaCl mM		N-Si mM				
		0	0.5	1	2	3
0	RL (cm)	9.41± 0.72	8.64± 1.59	9.60± 1.30	9.77± 0.94	10.14± 1.57
	FW (mg)	0.158± 0.006	0. 127*± 0.017	0.139*± 0.005	0.155± 0.015	0.151± 0.001
	DW (mg)	0.013± 0.001	0.012± 0.001	0.011± 0.001	0.011± 0.001	0.012± 0.002
150	RL (cm)	2.49 ♦♦± 0.58	3.24**♦♦± 0.78	4.39**♦♦± 0.66	3.41*♦♦± 0.93	5.07**♦♦± 2.37
	FW (mg)	0.124♦± 0.015	0.141*± 0.023	0.142*± 0.028	0.145*± 0.003	0.129♦± 0.011
	DW (mg)	0.014± 0.002	0.014± 0.004	0.012± 0.003	0.012± 0.001	0.011*± 0.003
200	RL (cm)	1.53♦♦± 0.43	1.91♦♦± 0.61	2.00*♦♦± 0.34	1.97*♦♦± 0.37	1.81*♦♦± 0.51
	FW (mg)	0.096♦♦± 0.014	0.110*♦♦± 0.004	0.119*♦♦± 0.007	0.120*♦♦± 0.005	0.120*♦♦± 0.028
	DW (mg)	0.014± 0.001	0.014± 0.002	0.013± 0.0001	0.011*± 0.001	0.011*± 0.0004

Values are the M (mean) ± SD (standard deviation) of three replications. (*) indicates significant effects at a 0.05 probability level, and (**) indicates significant effects at a 0.01 probability level, related to the NaCl150 or NaCl200 salinity treatments. (♦) indicates significant effects at a 0.05 probability level, and (♦♦) indicates significant effects at a 0.01 probability level, related to the non-treated control. RL = root length, FW = seedling fresh weight, DW = seedling dry weight, mg = milligram, cm = centimeter, and mM = millimolar

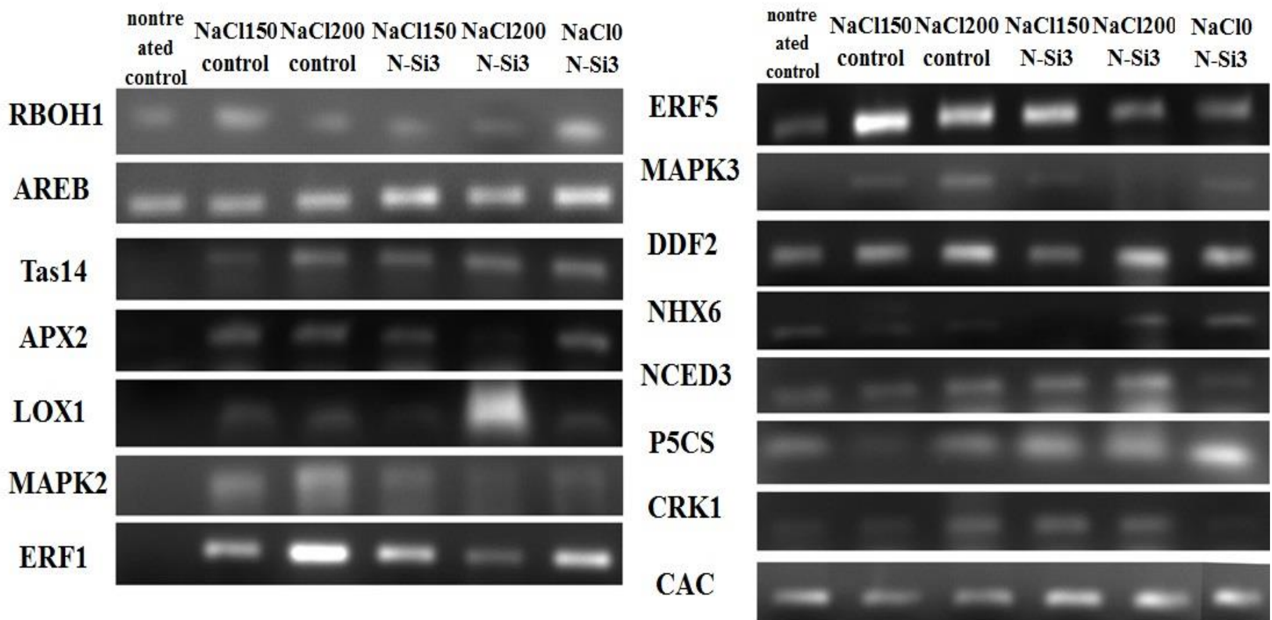

Fig 2. Semi-quantitative RT-PCR analysis of 14 salt stress genes expressed in tomato seedlings under two concentrations of NaCl, NaCl150 and NaCl200, and two treatment combinations of NaCl and N-Si, NaCl150 N-Si3 and NaCl200 N-Si3, in addition to N-Si3 alone and the non-treated control plants. The *CAC* gene was used as a positive control.

Table 3. Primer combinations used for semi-quantitative RT-PCR analysis. Fourteen tomato salt stress genes, in addition to the internal control gene, *CAC* were used.

Gene	Forward primer sequence 5’-3’	Reverse primer sequence 5’-3’	EST no. in SGN	Annealing Temp.	Product size (bp)
RBOH1	5’-CGGAGTCGGTATTGGTGGAG-3’	5’-AAGCCGAGTCTACACCGTTG-3’	Solyc08g081690.2.1	58°C	284
AREB	5’-ACAGGAGGGAGTGGTAAGGA-3’	5’-AGTCAAAGAGCCTTGCCTCT-3’	Solyc04g078840.2.1	56°C	159
TAS14	5’-CGTCGGAGGATGATGGAGAA-3’	5’-GTGTTCAATGCATCCCAGGG-3’	Solyc02g084850.2.1	58°C	168
APX2	5’-CCACTTGAGGGACGTGTTTG-3’	5’-CCCTTCCTTTTCCCCACTCA-3’	Solyc06g005150.2.1	56°C	187
LOX1	5’-TGTCTTTGGGTGGAATTGTGG-3’	5’-GGATTGCTCAGTTTCCCTTCC-3’	Solyc01g099190.2.1	56°C	217
MAPK2	5’-ACCACCTCAACGAGAAGCAT-3’	5’-TTGCTAGGCTTCAAGTCCCT-3’	Solyc08g014420.2.1	58°C	192
ERF1	5’-CAACAACAACAACAACAACATCA-3’	5’-TCCATTACGCGTTGAATCCC-3’	Solyc05g051200.1.1	52°C	179
ERF5	5’-GGAAGTTTGCAGCGGAGATT-3’	5’-ACCCTTTTACTGCCTGACGA-3’	Solyc03g093560.1.1	58°C	220
MAPK3	5’-TGGAATCGTCTGCTCTGTGT-3’	5’-GGCGGAGGAATCACATCTCT-3’	Solyc06g005170.2.1	56°C	174
DDF2	5’-GCCGAAATCTTCCGACCTTT-3’	5’-AGCTTCCACATGATCTCCCA-3’	Solyc03g026270.1.1	52°C	192
NHX6	5’-ACAAACCGCAGAAAAGCCTT-3’	5’-CCATGGCCGATTCTTCCAAG-3’	Solyc04g056600.2.1	54°C	158
NCED3	5’-GTATGGTTCACGCCGTTCAA-3’	5’-GCCTTGCAATTCCAGAGTGA-3’	Solyc07g056570.1.1	58°C	153
P5CS	5’-TCTTTACAGTGGTCCTCCCC-3’	5’-TATACGTTCCCCATGCAGCA-3’	Solyc08g043170.2.1	52°C	229
CRK1	5’-TGTATTCCTGCTCCTGGTGG-3’	5’-CTCCTGCAGCAAAATCCCTC-3’	Solyc03g112730.2.1	58°C	169
CAC	5’-CCTCCGTTGTGATGTAACTGG-3’	5’-ATTGGTGGAAAGTAACATCATCG-3’	Solyc08g006960.2.1	56°C	173

Forward and reverse primers designed from tomato salt stress genes sequences available on of SGN database` http://solgenomics.net/).

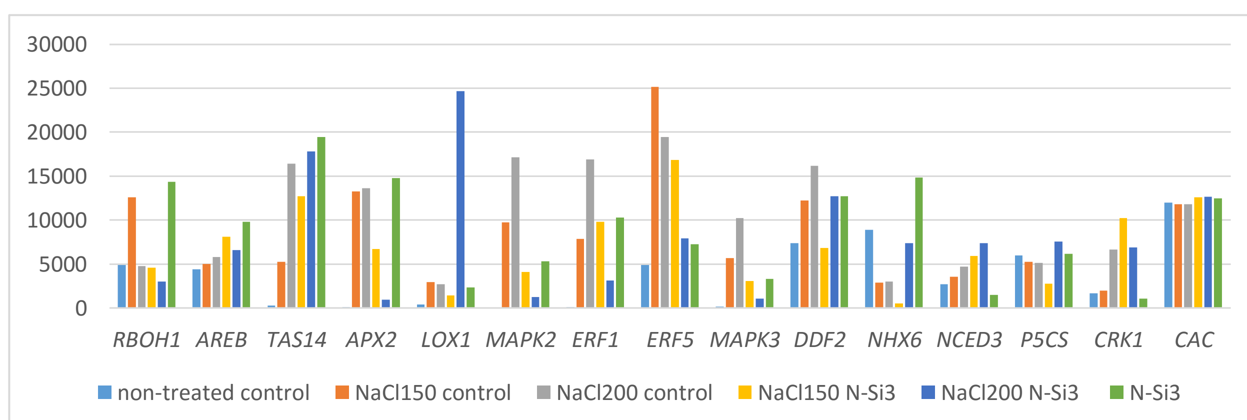

Fig 3. The relative expression level of 14 salt stress genes of tomato under two concentrations of NaCl, NaCl150 and NaCl200 and two treatment combinations of NaCl and N-Si, NaCl150 N-Si3 and NaCl200 N-Si3, in addition to N-Si3 alone and the non-treated control plants. The *CAC* gene was used as a positive control. The relative intensities values (*y*-axis) are an average of three independent experiments. The intensity of each band was measured with ImageJ software.

Two of the treatment combinations, NaCl150 N-Si3 and NaCl200 N-Si3, with their respective controls, NaCl150 and NaCl200, in addition to the N-Si3 treatment and the non-treated control, were chosen for the semi-quantitative RT-PCR analyses. As shown in Figs 2 and 3, the mRNAs for the *clathrin adapter complexes (CAC)* gene, used as a positive control, were expressed approximately uniformly in all the tested treatments and their controls. Analyses of the expression of the salt stress genes with semi-quantitative RT-PCR revealed different patterns of gene expression in response to the N-Si treatments, as demonstrated by the relative intensity of the gel bands.

Of the salt stress genes that were investigated in the tomato plants, the *TAS14*, *APX2*, *LOX1*, *MAPK2*, *ERF1* and *MAPK3* genes were upregulated in the NaCl stress and were not expressed in the non-treated plants. The same six genes also were expressed with N-Si treatment only, without NaCl, which means that these genes were induced also by N-Si. For all the investigated genes, the expression under NaCl150 stress was lower than that under NaCl200 stress, except for four genes, *RBOH1*, *LOX1*, *ERF5* and *P5CS*, which had higher expression under NaCl150 stress than under NaCl200 stress. The reduction in the expression of the *P5CS* gene under the NaCl150 level compared with the non-treated plants was similar to the expression of the *P5CS* gene in the soybean cultivar, Ataem-7, which was expressed less in this cultivar under the NaCl150 level than in the control. By contrast, the expression of the *P5CS* gene in another soybean cultivar, Üstün-1, was 4.44-fold higher than the expression in the control (Celik and Atak, 2012).

The expression of some genes, i.e., *AREB*, *TAS14*, *NCED3* and *CRK1*, was upregulated by N-Si under both levels of salt stress, NaCl150 and NaCl200. By contrast, the *RBOH1*, *APX2*, *MAPK2*, *ERF5*, *MAPK3* and *DDF2* genes were

downregulated with N-Si application under both levels of NaCl stress. The up- or downregulated genes with N-Si in this study were involved in regulating the responses of abscisic acid, which mediates the expression of a number of salt-responsive genes and acts in many ways as a mediator in the whole-plant response to salt stress (Iuchi et al., 2001; Sharma et al., 2005; Mahajan and Tuteja, 2005; Rossel et al., 2006; Yaish et al., 2010; Brock et al., 2010; Muñoz-Mayor et al., 2012; Cheng et al., 2013; Zhang et al., 2013; Zhou et al., 2014). The expression patterns of the six genes that were downregulated with N-Si under NaCl stress occurred because of their roles in catalysing the reduction of H_2O_2 to water (Zou et al., 2005; Zhang et al., 2009; Zhou et al., 2014; Yang, 2014; Mishra et al., 2009). This result was confirmed by Lei et al. (2008), who found that nanoparticles reduced antioxidant stress by reducing the concentration of H_2O_2 and increasing concentrations of some enzymes such as APX, which resulted in increased seed germination in some plant species. Previous studies confirmed that N-Si-induced oxidative stress triggered the activation of the antioxidant system through activation of the *MAPK* pathway (Guo et al., 2015). The *MAPKs* were also activated by silica nanoparticles in the dendritic immune cells of the mouse (Kang and Lim, 2012).

The other investigated genes, *LOX1*, *NHX6* and *P5CS*, were downregulated with N-Si under NaCl150, whereas these genes were upregulated with N-Si under NaCl200. Only the *ERF1* gene was upregulated with N-Si under NaCl150 and was downregulated under NaCl200. The *LOX1* pathway is involved in the control of gene expression by abscisic acid when exposed to abiotic stresses (Vicente et al., 2012). The *LOX1* also regulates the development of roots through control of the emergence of lateral roots through the production of (10E,12Z)-9-hydroperoxy-10,12-octadeca-dienoate (Vellosillo et al., 2007). The expression of the *NHX6* gene is regulated by *SOS2*, which activates directly and indirectly on the vacuolar membrane (Olías et al., 2009) and cooperates in ion homeostasis and cell expansion under normal conditions (Venema et al., 2003). The synthesis of proline is a frequent response to salt stress, and this osmolyte accumulates in the cytosol and provides proper osmotic adjustment. The proline is synthesized from glutamic acid through the action of the enzyme *P5CS*, which is also mediated by abscisic acid (Vinocur and Altman, 2005). The change in the molecular response to N-Si between the tested salinity levels indicated that N-Si altered the protective mechanisms under high levels of salinity. The plant adaptations to salinity are characterized with three distinct types: osmotic stress tolerance, Na+ or Cl− exclusion, and tolerance of tissues to an accumulation of Na+ or Cl− (Munns and Tester, 2008). Treatment with N-Si has improved the defence mechanisms of plants against salt stress toxicity by augmenting the transpiration rate, increasing water use efficiency, increasing total chlorophyll and proline content, and increasing carbonic anhydrase activity in the leaves of plants (Siddiqui et al., 2014).

Materials and Methods

Seed germination experiment

Tomato seeds, *Solanum lycopersicum* L., Castlerock VF variety from Pacific Seed Company, were used to evaluate the effects of N-Si on the germination traits of tomato under salinity stress. A factorial experiment was conducted based on a completely randomized design with three replications. The seed germination response of tomato plants to N-Si under salt stress was tested with seeds exposed to increasing concentrations of N-Si (0.5, 1, 2 and 3 mM) at two concentrations of NaCl (150 and 200 mM). The N-Si (silicon nanopowder, 99.99%, 20 nm) was purchased from U.S. Research Nanomaterials (Houston, TX).

The seeds were immersed in a 5% sodium hypochlorite solution for 10 min to ensure surface sterility (USEPA, 1996). The seeds were soaked in distilled water for 2 hrs and then in a series of prepared N-Si concentration suspensions for approximately 2 hrs. Seeds were then rinsed four times with distilled water. A piece of filter paper was placed into each of 100 mm × 15 mm Petri dishes, and 5 ml of a test solution was added. The seeds were transferred onto the filter paper, with 30 seeds per dish and a 3 mm or larger distance between each seed. The N-Si serial concentrations and the NaCl were added to Petri dishes along with 5 ml of each solution. Pure water used for the control treatment. The Petri dishes were covered, sealed with tape and incubated at room temperature. The germinating seeds were counted based on 2 mm of radical emergence 24 hrs after planting. The counting continued until the number of germinated seeds was constant for at least three days. The germination counting was halted after 8 days. From each dish, 10 seedlings were selected randomly for measurements of dry and fresh weights. The seed germination rate and the mean germination time were calculated, and the seedling dry and fresh weights and root length were measured.

Seed germination measurement

The final germination percentage was calculated based on the total number of germinated seeds at the end of the experiment. The measurements were conducted according to the International Rules for Seed Testing (ISTA, 1996). The germination indices were calculated using the following equations (Ellis and Roberts, 1981).

Germination percentage (GP%) = (Gf/n) × 100 (1)

Where; Gf = the total number of germinated seeds at the end of experiment, and n = the total number of seeds used in the test.

Mean germination time (MGT) = Σ NiDi/n (2)

Where; Ni = the number of germinated seeds until the ith day, Di = the number of days from the start of experiment until the ith counting, and n = the total number of germinated seeds.

Germination rate (GR) = Σ Ni/ΣTiNi (3)

Where; Ni = the number of newly germinated seeds at the time of Ti. = (a/1)+(b-a/2)+(c-b/3)+...+(n-n-1/N)

Semi-quantitative RT-PCR analysis

The expression of salinity stress genes was examined using semi-quantitative RT-PCR. Many genes from tomato have been identified and recorded in previous studies that were involved in salt tolerance. In this study, tomato salt stress genes were selected based on the availability of their ESTs sequence in SGN database (http://solgenomics.net/), a tomato genome database (SGN, 2015). These sequences were used to design the gene-specific primers.

The total RNA was extracted from tomato seedlings with TRIzol reagent (Sigma, USA), which was then reverse-transcribed into cDNA using the RT-PCR Superscript™ II reverse-transcriptase kit (Invitrogen, USA) with the Oligo(dT) primers. The EST sequences available in the SGN database were used to design gene-specific primers using Primer3 software (version v. 0.4.0) (http://frodo.wi.mit.edu/primer3/). The PCR was used to

amplify the cDNAs for each of the investigated tomato genes using combinations of forward and reverse primers with the synthesized cDNA as a template. Six templates were tested for the expression analysis of each salt stress gene, which included the following treatment combinations: the NaCl150 N-Si3 and NaCl200 N-Si3 treatments and their controls, the NaCl150 and NaCl200, the N-Si3 alone treatment and the non-treated control. The PCR reaction volume was 20 µl that contained 4 µl of 5X PCR ready mix that contained Taq polymerase (Solis BioDyne, Estonia), 0.4 µM of each primer, 3.5 µl of cDNA and sterile distilled water to a final volume of 20 µl. The amplifications were performed under the following conditions: an initial denaturation at 94°C for 5 min, then 35 cycles of denaturation at 94°C for 30 sec, annealing for 30 sec at a temperature that ranged from 52°C to 58°C (varied with each primer pair; see Table 3), and an extension at 72°C for 1.5 min. An extension at 72°C for 7 min was performed after all cycles were completed. The constitutive expressed gene, *CAC*, was used as positive control. The PCR products were separated on 1.2% agarose gel (Sigma, USA). The electrophoresis was then performed for 30 min at 100 volts in Tris/borate ethylenediaminetetraacetic acid (EDTA) buffer (TBE) (Sigma, USA). The products were stained with 3 µl of 10 mg/ml ethidium bromide (Sigma, USA) in 120 ml of TBA buffer. The stained bands in the gel were visualized and documented using a gel documentation system (Bio-Rad Laboratories, USA) with a UV light and Gel Doc 2000 Quantity One software. Each gene expression analysis was performed at least three times as independent PCR reactions and electrophoresis on gels, and one of the images is presented as a representative for each gene. The PCR products in the gel were quantified with the measurement of band intensity using ImageJ software, which generated a peak diagram for each band from which the area under each peak was determined as a reflection of band intensity.

Statistical analysis

The mean values and standard deviations were derived from three repeated samples of each treatment and the related control for seed germination and seedling growth measurements and from three independent PCR reactions for gene expression analysis. The data obtained from the various treatments were statistically analysed using *t*-tests at 0.01 and 0.05 levels of significance.

Conclusion

Salinity stress is a menace to agriculture and a major environmental factor that affects crops. Revealing gene networks that are affected in response to exposure to nanoparticles is the main aim of this study. This study focused on the effect of N-Si on salt tolerance mechanisms by examining expression profiling of salinity stress genes in tomato in the early stage of development. Our result indicated that exposure to N-Si concentrations alleviated the adverse effects of salt stress and improved germination percentage, germination rate, mean germination time, root length and seedling fresh weight of tomato seeds under NaCl stress. Four salt stress genes, *AREB*, *TAS14*, *NCED3* and *CRK1*, were upregulated by N-Si under salt stress, and six genes, *RBOH1*, *APX2*, *MAPK2*, *ERF5*, *MAPK3* and *DDF2*, were downregulated. Our results suggest that N-Si plays an important role in moderating inhibition in seed germination and plant growth in saline environments. We; therefore, hypothesize that tomato plants have a greater capacity for tolerating saline stress through the activation transcription of salt stress genes and its downstream machinery after N-Si supplementation.

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