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Original Paper

Low sodium chloride priming increases seedling vigor and stress tolerance to *Ralstonia solanacearum* in tomato

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Abstract Seed germination is the initial step of seedling development in plants. Seed priming with salts has been used to synchronise seed germination. In general, a long-term treatment with a relatively high salt concentration, such as 1 M NaCl, is employed. To improve the efficiency of this treatment, we examined the effect of seed priming with a lower NaCl concentration than conventional method in tomato (*Solanum lycopersicum*). Tomato seeds were soaked for 24 h at 25°C in the dark in 100–1000 mM of NaCl solution (NaCl-priming) or distilled water (hydro-priming). To estimate the effect of NaCl-priming on seed germination and subsequent seedling growth, the germination rate, seedling emergence, plant height, and hypocotyl and root length were investigated under NaCl-, hydro- and non-priming treatments. At 4 d after sowing, the seedling emergence was markedly promoted by 300 mM of NaCl-priming. The NaCl-priming also significantly enhanced the seed germination rate at 48 h after sowing. Seedling growth, as indicated by plant height, stem diameter and hypocotyl and root length, was promoted by NaCl-priming. These results suggest that priming with low saline has similar effects as conventional priming methods. A comprehensive gene expression analysis showed that the genes related to seedling growth and stress responses were up-regulated by NaCl-priming at 144 h after the start of the treatment, followed by advanced and uniform seed germination. The seedlings exhibited an increased tolerance to *Ralstonia solanacearum*, the causative agent of bacterial wilt of tomato, compared with the hydro-primed and non-primed seedling.

Key words: NaCl-priming, seedling emergence, seed germination, seed priming, stress tolerance.

Seed germination is the initial step in seedling development in plants. This step is defined as a series of events that commence with the uptake of water by a dry seed and that terminate with the elongation of the embryonic root, or radicle (Bewley and Black 1984). Generally, seeds initiate the germination process under favourable environmental conditions (i.e., sufficient or optimal water, oxygen and temperature). However, seeds under stressful conditions, such as salinity, drought, and extreme temperatures, show non-uniform germination and deficient seedling development. From the agricultural aspect, the uniform germination and consequent normal growth of seedlings is important to disease protection and yield as well as the quality of the crop. For many crops, non-uniform and/or delayed germination could result in diminished seedling quality. It is also important to establish seedlings that will grow vigorously under stressful conditions such as low water and salinity.

To improve seed germination rates and field emergence under adverse environmental conditions, the pretreatment of seeds, called seed priming, has been employed at the site of agriculture. Appropriate priming treatment synchronises germination and improves seed performance in many crop species (Bradford 1986; Heydekker et al. 1973). Because certain germination processes are initiated before sowing, seed priming generally results in more rapid germination and field emergence under adverse germination conditions (McDonald 2000). Pretreatment with an abiotic stress, such as sodium chloride (NaCl), prior to sowing enhances the tolerance of plants to salinity (Strogonov 1964).

Various seed priming methods have been adopted for tomato seeds since the 1980s. Tomato seeds primed with 3% KNO_3 or polyethylene glycol (PEG) 8000 for 7 d (Alvarado et al. 1987) or 120 mM K_2HPO_4 and 150 mM KNO_3 for 5 d (Argerich et al. 1989) exhibited higher

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germination and seedling emergence ratios and initial growth than non-primed seeds. However, because of the cost and labour, of priming methods, they have not yet been accepted. However, NaCl has been used as a low-cost reagent for seed priming. It has also been reported that priming with 1 M NaCl for 36 h and 6 M NaCl for 3 d resulted in increased a higher yield and field emergence (Cano et al. 1991; Cayuela et al. 1996). Furthermore, pretreatment with NaCl enhances the salt tolerance of plants (Strogonov 1964). However, in these previous reports, much higher concentrations and much longer treatment periods were needed for NaCl-priming than in our studies with NaCl.

However, the mechanisms for seed priming that trigger the aforementioned changes in the processes of germination and seedling growth are not fully understood. Although transcriptional analyses from seed priming to initial germination were conducted with early stages of germinating seeds under the priming treatment in several plant species, such as, *Brassica oleracea*, Arabidopsis, and tomato (Auge et al. 2009; Bassel et al. 2008; Soeda et al. 2005), a gene expression profile in the seedling several days after priming treatment has not been reported. Moreover, it is still unclear how the priming treatment confers biotic stress tolerance to primed plants.

In this study, we optimised NaCl-priming with a lower concentration than the conventional methods in an effort to establish a more effective and low-cost priming method in the tomato. We ensured that a 300 mM NaCl treatment for 24 h was effective for uniform germination and rapid seedling growth. We recently observed that the priming treatment confers an increased tolerance to *Ralstonia solanacearum*, which is the causative agent of bacterial wilt in tomatoes. For further insight into the phenomenon, we investigated the gene expression profile in NaCl-primed seeds/seedlings and discussed the mechanisms of priming that caused those changes in the seedlings.

Materials and methods

Plant materials and priming treatments

Tomato seeds (*Solanum lycopersicum* L. cv. 'Momotaro Haruka') (Takii & Co., Ltd.) were used for all experiments. The seeds were soaked for 24 h at 25°C in the dark in a 300 mM NaCl solution (NaCl-priming) or distilled water (hydro-priming). After the priming treatments, the seeds were washed with distilled water and then submitted to the germination and field emergence tests described below. Non-primed seeds were used as a control.

Field emergence test

The field emergence test was repeated five times with three replications for each trial. For each trial, 24 seeds were submitted using a different seed lot. After the priming treatment, the seeds were sown on a 200-cell plug tray filled with commercial culture soil (Peatpot P; Hokkaido Peatmoss Co., Ltd). The plug trays were maintained in an incubator at 25°C and approximately 85% humidity in the dark. After the seedlings began to emerge, the trays were transferred to a growth chamber at 23/15°C (light/dark) under 16 h light/8 h dark conditions. The seedlings were watered once every other day. The number of emerged seedlings was counted at 24-h intervals for 5 d after the onset of seedling emergence. We defined a seedling as 'emerged' when the hypocotyl visibly protruded on the surface of the soil. Plant height and stem diameter were measured at 3 and 4 weeks after sowing, respectively.

Germination test

The germination test was repeated seven times with three replications for each trial. For each trial, 25 seeds were submitted using a different seed lot. After the priming treatment, the seeds were placed on two-layered filter papers (ADVANTEC No. 2; Toyo Roshi Kaisha, Ltd) in a covered plastic case (19×13×6.5 cm). The seeds were moistened with 15.5 ml (normal condition) or 3.9 ml (one-quarter of the normal condition) of distilled water and 25 mM or 50 mM NaCl solution. The plastic cases were maintained in an incubator at 25°C in the dark. The number of germination events was counted at 6-h intervals from the onset of germination. We defined a seed as 'germinated' when the radicle protruded through the seed coat. The hypocotyl length was measured at 5 d after sowing. Additionally, to measure the root length, the seeds were placed on 1.5% agar in a test tube (1.8 diameter×18 cm) and maintained in the incubator at 25°C in the dark. Subsequently, the hypocotyl length was measured at 60 h after sowing.

RNA isolation and Microarray analysis

After the germination test, total RNA was extracted from the NaCl-primed and hydro-primed seedlings at 5 d after sowing using the RNeasy Plant Mini kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. The RNA concentration and quality were determined using an Agilent Bioanalyser 2100 (Agilent RNA 6000 Nano Kit, Germany). The extracted RNA was dissolved in RNase free water, and stored at −80°C until further use. The comprehensive gene expression analyses were performed with the tomato 44 k DNA microarray designed by Imanishi et al. (2005) based on the platform established by Agilent Technologies (60 mer, 44 k×4). The genes included in the array were derived from the unigene set, ver. 11.0, June 2006 released from the Institute for Genomic Research (TIGR). All microarray procedures, including RNAs labelling, hybridisation and scanning and data analyses, were performed according to Shimono et al. (2007). The data were normalised and statistically analysed using the GeneSpring GX 7.3 software (Agilent). The error model "maximum of averaged and Bayesian error variances" was used to reduce

false positives. Measuring the false discovery rate controlled the false positives. On the basis of the TC number in TIGR or the accession number in GenBank, the annotation was obtained by a homology search against TBLASTX on DDBJ.

Plant growth conditions and inoculation

After measurement in the field-emergence test, the seedlings were transplanted to a pot (diameter 6.5×5.7 cm) filled with commercial medium that was used in the field emergence test at 4 weeks after sowing and subjected to inoculation with *Ralstonia solanacearum* (race 1), which was kindly provided by the National Institute of Vegetable and Tea Science, Japan. To prepare the inoculums, the bacteria were cultured in BG broth medium (1% Bacto Peptone, 0.1% casamino acid, 0.1% yeast extract and 0.5% glucose) for 24 h at 35°C. The population of bacteria were measured on triphenyl tetrazolium chloride (TTC) agar medium (1% Bacto Peptone, 0.1% casamino acid, 0.5% glucose 1.8% agar and 0.005% TTC). A 2 μl volume of the bacterial suspension, adjusted to approximately 2.0×10^7 CFU ml^{-1} (colony-forming unit per ml), was inoculated onto the cut surface of the petiole of the first leaf above the cotyledon, which was excised at 5 mm from its stem. As a control, the same volume of distilled water was applied onto other individuals. After the inoculation, the plants were incubated at 30°C for 2 weeks, and the tolerance to *R. solanacearum* was estimated by checking for wilted leaves. Plants with more than 25% wilted leaves were defined as 'susceptible'.

Results

Field-emergence and subsequent growth of seedlings

Prior to this work, we had optimized NaCl concentrations for priming among 100 mM and 1000 mM and treatment duration between 12–24 h (Figure S1, S2). Although the eventual ratio of the field emergence was almost similar among the tested conditions, 300 mM of NaCl for 24 h was most effective in the initial rise. Therefore, we utilized this condition, 300 mM for 24 h, for subsequent analyses. As shown in Figure 1, the period required for the initial field-emergence and the final percentage of the emergence were not different among the NaCl-, hydro- and non-primed seeds. However, the rate of the initial increase of emergence was highest in NaCl-primed seeds at 4 and 5 d after sowing. The periods required for reaching 75% of the emergence were 4 d in NaCl-primed, and 5 d in hydro-primed and non-primed seeds, respectively.

To examine the effects of the priming treatment on subsequent seedling growth, the plant height and stem diameter were measured. Both the plant height and the stem diameter slightly but significantly increased in the NaCl-primed seedlings compared with the hydro-primed and non-primed seedlings (Figure 2).

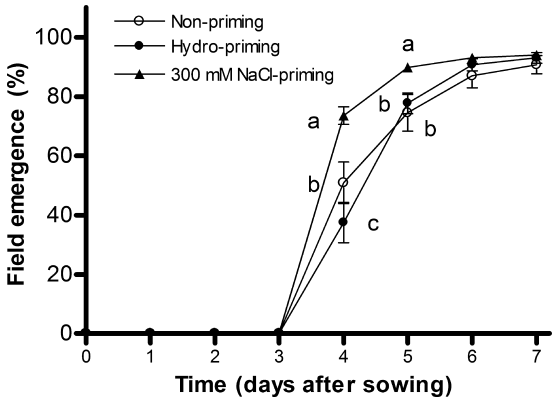

Figure 1. Seedling emergence ratio of primed and non-primed tomato seeds. Open circles, closed circles and closed triangles indicate non-primed, hydro-primed and NaCl-primed seeds, respectively. Hydro-primed and NaCl-primed seeds were soaked for 24 h at 25°C in the dark in distilled water or a 300 mM NaCl solution before sowing. Seeds were sown in a 200-cell plug tray containing a commercial medium. Values are given as the average±SE. Values followed by different letters at each time point are significantly different ($P<0.05$) according to Tukey's test.

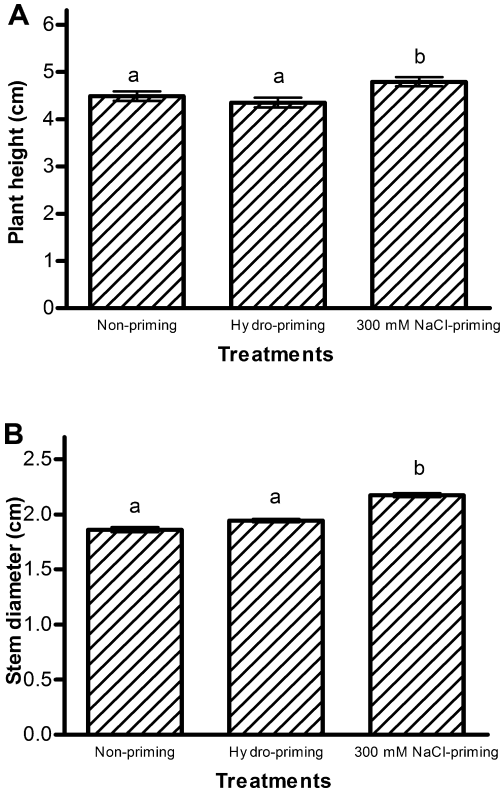

Figure 2. Plant heights and stem diameters of the primed and non-primed tomato seedlings. Plant height and stem diameter was measured approximately 3 and 4 weeks after sowing, respectively. The values represent the averages±SE ($n>59$). Error bars accompanied by different letters are significantly different ($P<0.01$) according to Tukey's test.

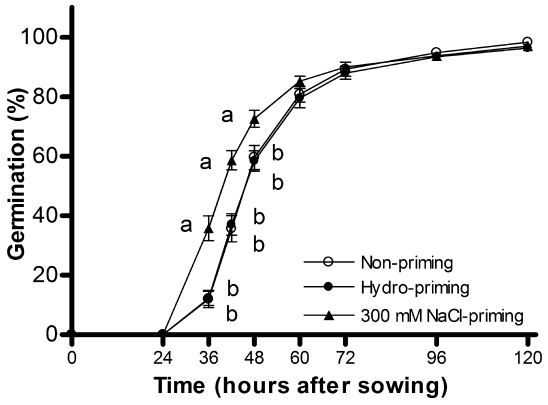

Figure 3. Germination percentages of primed and non-primed tomato seeds. Open circles, closed circles and closed triangles indicate non-primed, hydro-primed and NaCl-primed seeds, respectively. Hydro-primed and NaCl-primed seeds were soaked for 24 h at 25°C in the dark in distilled water or a 300 mM NaCl solution before sowing. The seeds were sown on filter paper moistened with 15.5 ml of distilled water and placed in a covered plastic case. Values are means±SE. Values followed by different letters at each time point are significantly different ($P<0.05$) according to Tukey's test.

Germination ratio and subsequent growth of seedlings

To further investigate the effects of the priming treatment, we also examined the germination ratio of the primed seedlings (Figure 3). Similar to the field emergence, the period required for of the initiation of germination and the final germination ratio were not different among the 300 mM NaCl-, hydro- and non-primed seeds. However, the rate of the initial increase in germination was significantly higher for the NaCl-primed seeds from 36 to 48 h after sowing. To examine the effects of the priming on early seedling growth after germination, the hypocotyl and root length were measured (Figure 4, S3). Both the hypocotyl and the root length were significantly longer in the NaCl-primed seedlings as compared with the hydro-primed and non-primed seedlings (Figure 4). This tendency was especially emphasised in the root length.

Gene expression profiles in the primed seedlings

To profile the gene expression predominantly induced by the 300 mM NaCl-priming treatment, a microarray analysis was conducted using the primed seedlings at 5 d after sowing. Compared with the hydro-primed seedling, a total of 4.4% of the genes were up-regulated greater than 2-fold by NaCl-priming. For instance, the expression of the genes most likely related to the promotion of plant growth through auxin signalling and abiotic and biotic stresses was increased as shown in Table 1. Transcripts for the transport inhibitor response (*TIR*) -like protein, *LeTIR* and auxin response factor (*SlARF8* and *SlARF3*) related to auxin signalling were increased by 4.3, 2.3, 2.0 and 2.0 times, respectively,

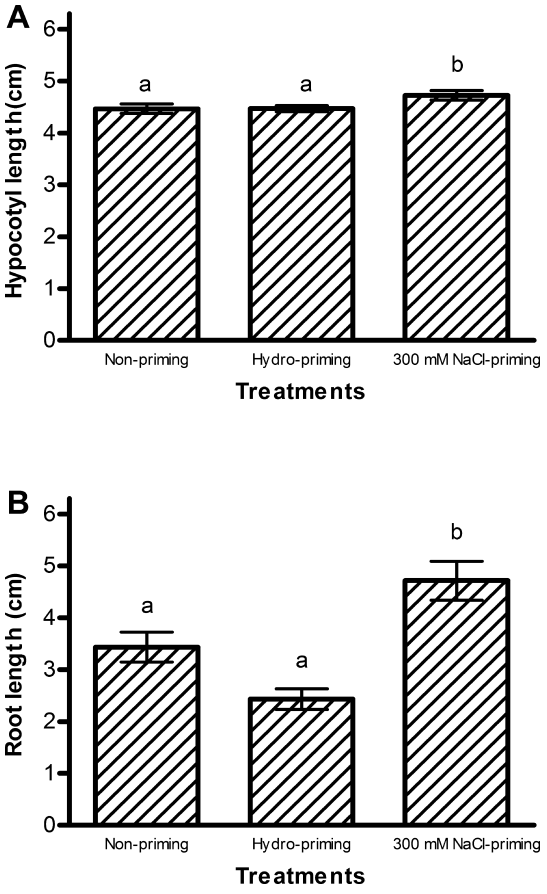

Figure 4. Hypocotyl and root lengths of primed and non-primed tomato seeds. Hypocotyl and root length were measured approximately 5 and 3 d after sowing. The values are means±SE. Values followed by different letters at each time point are significantly different ($P<0.05$) according to Tukey's test.

compared to their expression in the hydro-primed seeds. Similarly, the endo-1,4-beta-gulucanase (*SlCel4*) precursor and xyloglucan endotransglycosylase/hydrolase 10 (*SlXTH10*) related to plant growth were increased 2.6 and 2.3 times, respectively. The gene expression of proteins related to abiotic stress, such as 14-3-3 protein 7 (*SlTFT7*) and cytosolic ascorbate peroxidase 1 (*SlApx*), was increased to 4.1 and 4.0 times, respectively. However, for biotic stress, the expression of a gene that is homologous to the resistance genes for bacterial wilt was not observed. However, the transcripts for pathogenesis-related (PR) proteins 2, 3 and 5 and the DNA-binding protein 3 (*tWRKY3*) increased by 5.1, 2.2, 2.1 and 4.1 times, respectively. In addition, the expression of the genes related to signal transduction in plants, such as kinases, phosphatases and transcription factors, heat shock proteins and others, are also shown in Table S1.

Germination under stressful conditions

Because a number of genes related to the abiotic stress response were up-regulated in the 300 mM NaCl-primed seedling (Table 1), the tolerance of the primed seed to

Table 1. Genes up-regulated more than 2-fold by NaCl-primed compared to the hydro-primed seedling

Systematic	Fold change	TC/GB number	Score	E Value	Annotation	*Source*
Growth and auxin related candidates						
TowgeN_I_28637	5.2	TC188446	125	2e-35	Auxin/indole-3-acetic acid 4 (IAA4)	*Solanum tuberosum*
TowgeN_I_19099	4.3	TC178908	1403	0.0	TIR1-like protein	*Solanum lycopersicum*
TowgeN_I_15553	4.0	TC175362	289	7e-90	EF-1-alpha-related GTP-binding protein	*Nicotiana tabacum*
TowgeN_I_23908	3.3	TC183717	406	e-123	Developmentally ragulated GTP binding protein 1	*Pisum sativum*
TowgeN_I_13191	3.2	TC173000	246	5e-64	ARGOS	*Solanum lycopersicum*
TowgeN_I_10054	3.2	TC169863	1685	0.0	Aplpha-L-arabinofuranosidase	*Solanum lycopersicum*
TowgeN_I_11540	3.1	TC171349	213	5e-53	GTP-binding protein	*Arabidopsis thaliana*
TowgeN_I_49709	3.1	BG140061	157	8e-37	Glucan endo-1,3-beta-glucosidase 5 precursor	*Zea mays*
TowgeN_I_30127	3.0	TC189936	320	9e-86	Kinesin-like protein	*Arabidopsis thaliana*
TowgeN_I_14525	2.6	TC174334	1002	0.0	Endo-1,4-beta-glucanase (Cel4) precursor	*Solanum lycopersicum*
TowgeN_I_22405	2.5	TC182214	780	0.0	IAA-Ala hydrolase (IAR3)	*Arabidopsis thaliana*
TowgeN_I_26496	2.5	TC186305	241	5e-90	Cellulose synthase Z632	*Zinnia elegans*
TowgeN_I_22769	2.4	TC182578	223	2e-56	Growth-regulating factor 3	*Oryza sativa*
TowgeN_I_11661	2.4	TC171470	946	0.0	Expansin 1 (EXP1)	*Solanum lycopersicum*
TowgeN_I_21489	2.4	TC181298	408	e-112	Exo-1, 3-beta-glucanase	*Lilium longiflorum*
TowgeN_I_20995	2.3	TC180804	1045	0.0	LeTIR	*Solanum lycopersicum*
TowgeN_I_10162	2.3	TC169971	1606	0.0	XYloglucan endotransglycosylase/hydrolase 10 (XTH10)	*Solanum lycopersicum*
TowgeN_I_29262	2.2	TC189071	101	2e-62	Extensin	*Solanum tuberosum*
TowgeN_I_25303	2.2	TC185112	171	2e-40	Kinesin-like protein	*Arabidopsis thaliana*
TowgeN_I_27778	2.2	TC187587	347	e-123	Katanin	*Arabidopsis thaliana*
TowgeN_I_29924	2.1	TC189733	172	3e-40	Cell wall protein Expansin1 precursor	*Mirabilis jalapa*
TowgeN_I_12797	2.1	TC172606	304	1e-92	2,4-D inducible glutathione S-transferase	*Glycine max*
TowgeN_I_22049	2.1	TC181858	103	3e-38	Lectin-like protein	*Arabidopsis thaliana*
TowgeN_I_20888	2.1	TC180697	471	e-130	Putative polygalacturonase	*Arabidopsis thaliana*
TowgeN_I_16969	2.1	TC176778	895	0.0	Alpha tubulin	*Nicotiana tabacum*
TowgeN_I_21293	2.0	TC181102	737	0.0	Auxin response factor 8 (ARF8)	*Solanum lycopersicum*
TowgeN_I_23335	2.0	TC183144	254	e-150	Putative beta-galactosidase BG1	*Vitis vinifera*
TowgeN_I_22422	2.0	TC182231	175	1e-89	SKIP interacting protein 26	*Oryza sativa*
TowgeN_I_15438	2.0	TC175247	5475	0.0	Auxin response factor 3 (ARF3)	*Solanum lycopersicum*
Abiotic stress related candidates						
TowgeN_I_21112	5.5	TC180921	190	4e-46	Drought responsive element binding protein	*Vitis vinifera*
TowgeN_I_49348	5.3	AW398615	1140	0.0	Violaxanthin de-epoxidase	*Solanum lycopersicum*
TowgeN_I_14813	4.5	TC174622	481	e-134	Alpha/beta fold family protein	*Solanum lycopersicum*
TowgeN_I_10438	4.1	TC170247	686	0.0	14-3-3 protein 7 (TFT7)	*Solanum lycopersicum*
TowgeN_I_21803	4.0	TC181612	753	0.0	Cytosolic ascorbate peroxidase 1 (Apx1)	*Solanum lycopersicum*
TowgeN_I_14509	3.9	TC174318	889	0.0	Ser/Thr specific protein phosphatase 2A B regulatory subunit alpha isoform	*Medicago sativa subsp.* x *varia*
TowgeN_I_31243	3.7	TC191052	229	4e-90	Fructose-6-phosphate 2-kinase	*Bruguiera gymnorhiza*
TowgeN_I_15870	3.2	TC175679	311	e-160	Ser/Thr specific protein phosphatase 2A B regulatory subunit beta isoform	*Medicago sativa subsp.* x *varia*
TowgeN_I_20253	3.1	TC180062	210	e-116	ERD7	*Arabidopsis thaliana*
TowgeN_I_49259	3.0	AI487805	125	5e-27	Salt inducible protein kinase	*Zea mays*
TowgeN_I_33641	2.9	BE435108	111	5e-51	Protein phosphatase 2C	*Nicotiana tabacum*
TowgeN_I_46251	2.7	AW944845	149	7e-35	Dehydration-induced protein ERD15	*Solanum lycopersicum*
TowgeN_I_21031	2.5	TC180840	385	e-169	Dehydrin TAS14	*Solanum lycopersicum*
TowgeN_I_14161	2.5	TC173970	351	0.0	NAC domain protein NAC2	*Solanum tuberosum*
TowgeN_I_49356	2.4	AW398708	232	4e-59	Chaperonin 60 alpha chain-like protein	*Arabidopsis thaliana*
TowgeN_I_15813	2.4	TC175622	487	0.0	Protein phosphatase 2C (PP2C)	*Fagus sylvatica*
TowgeN_I_31099	2.1	TC190908	308	e-146	Membrane-associated salt-inducible protein	*Nicotiana tabacum*
TowgeN_I_49210	2.1	BE459326	142	9e-33	Zeta class glutathione transferase GSTZ1	*Populus trichocarpa*
TowgeN_I_48111	2.1	AW933595	74	3e-12	UV-damaged DNA binding protein 1	*Solanum lycopersicum*
TowgeN_I_17747	2.1	TC177556	52	6e-10	Dehydrin like protein	*Solanum sogarandinum*

Table 1. Genes up-regulated more than 2-fold by NaCl-primed compared to the hydro-primed seedling

Systematic	Fold change	TC/GB number	Score	E Value	Annotation	*Source*
Biotic stress related candidates						
TowgeN_I_27314	5.6	TC187123	668	0.0	Viroid symptom modulation protein	*Solanum lycopersicum*
TowgeN_I_29180	5.1	TC188989	957	0.0	Beta-1,3-glucanase (PR2)	*Solanum lycopersicum*
TowgeN_I_10011	4.6	TC169820	1475	0.0	Disease resistance gene homolog Mi	*Solanum lycopersicum*
TowgeN_I_36825	4.2	AW616209	247	1e-63	Wound induced protease inhibitor (WIPI)	*Solanum lycopersicum*
TowgeN_I_14188	4.1	TC173997	37	5e-07	DNA-binding protein 3 (tWRKY3)	*Nicotiana tabacum*
TowgeN_I_28506	3.8	TC188315	45	2e-18	Resistance protein XiR1. 2 (NBS-LRR)	*Vitis arizonica*
TowgeN_I_26806	3.5	TC186615	1112	0.0	Avr9/Cf-9 rapidly elicited protein 189	*Nicotiana tabacum*
TowgeN_I_11971	3.5	TC171780	93	4e-33	KED	*Nicotiana tabacum*
TowgeN_I_16413	3.3	TC176222	362	e-103	Cytochrome P450 A	*Capsicum annuum*
TowgeN_I_23792	3.2	TC183601	152	2e-47	P-rich protein EIG-I30	*Nicotiana tabacum*
TowgeN_I_38771	2.9	AI772130	103	1e-19	Resistance protein-like protein (NBS-LRR)	*Solanum lycopersicum*
TowgeN_I_20335	2.9	TC180144	1820	0.0	14-3-3 protein 5 (TFT 5)	*Solanum lycopersicum*
TowgeN_I_16852	2.9	TC176661	287	8e-75	PR-1 protein	*Solanum lycopersicum*
TowgeN_I_10527	2.8	TC170336	622	e-176	Cathepsin D inhibitor protein	*Solanum lycopersicum*
TowgeN_I_49644	2.6	AI771377	160	3e-38	GRAS7	*Solanum lycopersicum*
TowgeN_I_12541	2.6	TC172350	158	3e-53	bZIP transcription factor (PPI1)	*Capsicum chinense*
TowgeN_I_24241	2.6	TC184050	55	1e-05	Pto-responsive gene 1 protein	*Solanum lycopersicum*
TowgeN_I_30522	2.5	TC190331	117	1e-48	Proteinase inhibitor I precursor	*Solanum tuberosum*
TowgeN_I_49434	2.4	AI486657	240	8e-75	Gamma-thionin	*Solanum lycopersicum*
TowgeN_I_14249	2.4	TC174058	96	4e-22	bZIP transcription factor AtbZIP5	*Arabidopsis thaliana*
TowgeN_I_10113	2.3	TC169922	1078	0.0	Mitogen-activated protein kinase 1	*Solanum lycopersicum*
TowgeN_I_19985	2.2	TC179794	674	0.0	Hypersensitive-induced reaction protein	*Capsicum annuum*
TowgeN_I_27804	2.2	TC187613	247	5e-97	Class IV chitinase (PR3)	*Nicotiana tabacum*
TowgeN_I_51097	2.2	BG629139	84	4e-15	Defense-signaling glycopeptide hormone precursor	*Solanum lycopersicum*
TowgeN_I_18641	2.2	TC178450	683	0.0	CMV 1a interacting protein 1	*Nicotiana tabacum*
TowgeN_I_22503	2.1	TC182312	118	7e-47	CC-NBS-LRR type resistance protein	*Capsicum chinense*
TowgeN_I_10028	2.1	TC169837	707	0.0	PSTVd RNA-biding protein (Virp1)	*Solanum lycopersicum*
TowgeN_I_13179	2.1	TC172988	231	e-106	ACRE 132-like protein	*Solanum tuberosum*
TowgeN_I_11234	2.1	TC171043	415	e-114	24K germin like protein	*Nicotiana tabacum*
TowgeN_I_21732	2.1	TC181541	829	0.0	14-3-3 protein 4 (TFT 4)	*Solanum lycopersicum*
TowgeN_I_10121	2.1	TC169930	961	0.0	MAP kinase kinase	*Solanum lycopersicum*
TowgeN_I_12426	2.1	TC172235	123	3e-56	Thaumatin-like protein (PR5)	*Arabidopsis thaliana*
TowgeN_I_48805	2.0	AW220676	295	4e-78	Receptor-like kinase	*Brassica oleracea*

The genes likely related to the promotion of plant growth through auxin signalling and abiotic and biotic stresses are shown.
* 'Fold change' indicates changes in the expression levels in NaCl-primed seedlings compared to the hydro-primed seedlings.
** 'TC' and 'GB' indicate the accession number on TIGR and GenBank, respectively.

abiotic stresses, such as drought and salinity, during germination was estimated (Figure 5). The primed and non-primed seeds were not significantly different from each other in terms of the final germination ratio (Figure 5A, B, C). Under drought-stress conditions, the time required for the initiation of germination increased (Figure 3, 5A). However, the germination ratio for the 300 mM NaCl-primed seeds was significantly higher than that of the non-primed and hydro-primed seeds from 48 to 72 h after sowing (Figure 5A). Under salinity conditions of 25 mM or 50 mM NaCl, the time required for to begin germination was delayed, and the germination ratio was decreased at the beginning of germination compared to normal conditions (Figure 3, 5B, C). The germination ratio of NaCl-primed seeds was significantly higher than that of the non-primed and hydro-primed seeds from 42 to 48 h and 42 to 60 h after sowing under the 25 mM and 50 mM of NaCl stresses, respectively (Figure 5B, C). These observations suggest that NaCl-priming affects drought and salinity stress conditions.

Tolerance to bacterial wilt

Because a number of genes related to the biotic stress response were also up-regulated in the 300 mM NaCl-primed seedling (Table S1), the inoculation trial of the bacterial wilt disease that widely affects the Solanaceae was performed. To avoid the promotion of root growth by NaCl-priming (Figure 4B), the bacterial wilt was inoculated directly into the petiole of the first leaf. One week after inoculation, the disease symptoms began to appear in each treatment (Figure 6). The ratio of the wilted plants and the infection speed in NaCl-primed plants was lower and slower than that of the non-primed

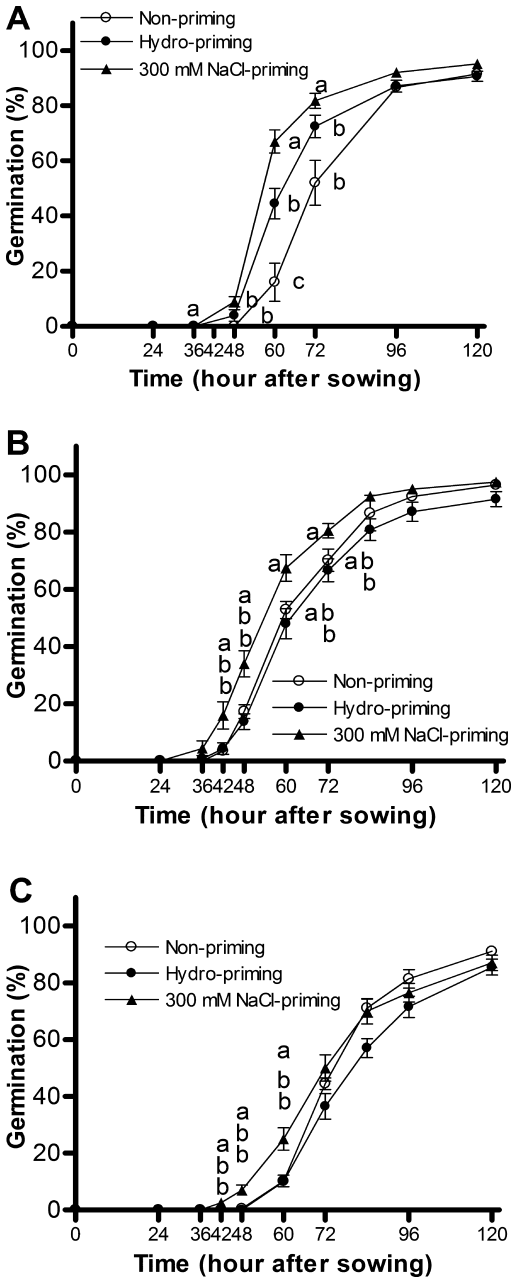

Figure 5. Germination percentages of primed and nonprimed tomato seeds under stress conditions. Open circles, closed circles and closed triangles indicate non-primed, hydro-primed and 300 mM NaCl-primed seeds, respectively. (A) Filter paper in a covered plastic case was moistened with 3.9 ml of distilled water (one-quarter of the volume used in the test shown in Figure 3). Filter paper moistened with 15.5 ml of a 25 mM or 50 mM NaCl solution was used in (B) and (C), respectively. The values represent the averages±SE. Values followed by different letters at each time point are significantly different ($P<0.05$) according to Tukey's test.

and hydro-primed plants (Figure 6), demonstrating an increased tolerance of seedling produced from NaCl-primed seeds to the bacteria wilt.

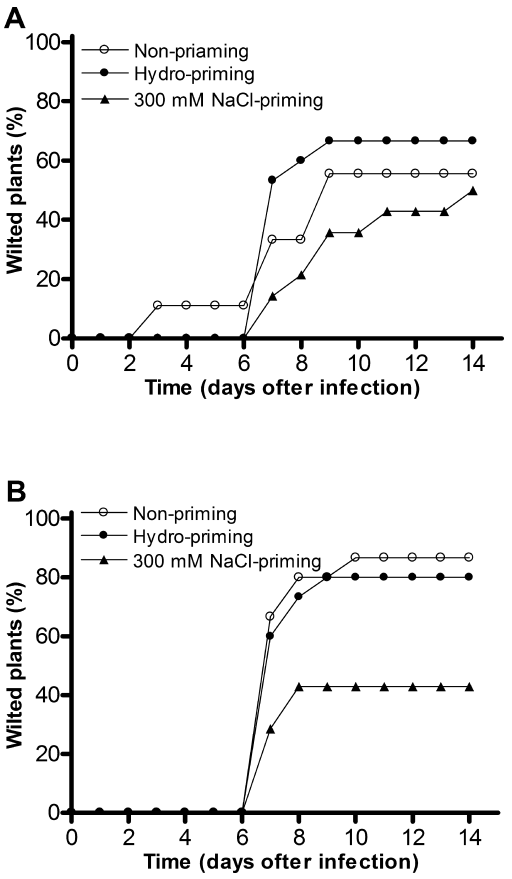

Figure 6. Response of seedlings established from primed and non-primed tomato seeds to *Ralstonia solanacearum*. Open circles, closed circles and closed triangles indicate non-primed, hydro-primed and 300 mM NaCl-primed seeds, respectively. Wilted plants were defined as plants that showed more than 25% of leaves with wilted symptoms. Two independent experiments were performed in (A) and (B) using at least 13 plants for each treatment.

Discussion

Seed-priming techniques have been used to improve both seed germination and seedling emergence and have been adapted for tomato (Alvarado et al. 1987; Argerich et al. 1989; Cano et al. 1991; Cayuela et al. 1996). In this study, tomato seeds primed with a lower NaCl concentration than those used in previous reports showed a more rapid germination and uniform seedling emergence than the non-primed and hydro-primed seeds. The eventual germination ratio in the present results was not so different from the previous reports using much higher salt concentrations (Figure S1). However, in this study, we demonstrated that the lower NaCl-priming is effective enough to promote seed vigor as well as germination in tomato. Additionally, the 24 h of treatment duration which we adopted in this work is actually shorter compared to the 36 h–6 d of duration in conventional methods (Alvarado et al. 1987; Argerich et al. 1989; Cano et al. 1991; Cayuela et al. 1996), indicating our method would contribute to save time as well as cost

for priming.

One possible explanation for the early field emergence of the 300 mM NaCl-primed seeds is the promotion of seed germination by NaCl treatment. The process of germination in tomato seeds has been studied in some detail. The tomato embryo is surrounded by a rigid endosperm. When germination begins, as a first step, the endosperm that encloses the radicle tip, and the endosperm cap weakens to allow the radicle to emerge (Groot and Karrssen 1987). Enzymes, such as endo-β-mannanase, expansin, β-1,3-glucanase and xyloglucan endotransglycosylase, are thought to be involved in the weakening of the endosperm cap. Gibberellin (GA) induces the transcription of those genes. However, except for β-1,3-glucanase, the expression of the enzymes and the concomitant weakening of the endosperm cap are not inhibited by abscisic acid (ABA), although radical emergence is inhibited by ABA (Chen and Bradford 2000; Chen et al. 2002; Nonogaki et al. 2000; Wu et al. 2001). In addition, it has been proposed that the weakening of the tomato endosperm cap is a biphasic process (Toorop et al. 2000). The first step is characterised by an ABA-independent endosperm cap weakening that is associated with enzymes, such as endo-β-mannanase. During the second step, ABA inhibits germination. Therefore, the enzymes responsible for the weakening of the endosperm cap and a process related to germination are regulated by plant hormones, such as ABA and GA. To increase our understanding of the effect of NaCl-priming on germination, a further investigation of the how the plant hormones function in the germinating seed to regulate the expression of genes encoding the enzymes required for endosperm cap weakening under priming treatments is needed.

The potential improvement of seedling growth by seed priming is widely recognised. Throughout vegetative growth, the seedlings primed with 3% KNO_3 or PEG 8000 for 7 d maintained a greater average dry weight, leaf area and ground cover percentage than those of the non-primed seedlings (Alvarado et al. 1987). Furthermore, those attributes were entirely due to early emergence, rather than to an increased growth rate (Alvarado et al. 1987). In our study, the plant height and stem diameter of seedlings primed with 300 mM NaCl were significantly higher and larger than those of the non-primed or hydro-primed seedlings (Figure 2). Similarly, the hypocotyl and root length were also longer than those with non-primed or hydro-primed seedlings at early growth stages (Figure 4). Moreover, the field emergence and germination ratio were also promoted by NaCl-priming (Figure 1, 3). Thus, our results are consistent with the previous reports, suggesting that the growth promotion results from improved seedling emergence and germination by NaCl-priming.

Auxin participates in many aspects of the developmental response in plants, including growth, cell expansion and differentiation (Friml 2003). The results of the gene expression profile indicated an up-regulation of the genes related to auxin signalling, such as *TIR-like protein*, *LeTIR*, *SlARF8* and *SlARF3*, in the 300 mM NaCl-primed seeds (Table 1). It has also been reported that the auxin concentration is increased in the NaCl-treated tomato root (Albacete et al. 2008), suggesting the increased auxin concentration in NaCl-primed seeds/seedlings and the subsequent up-regulation of auxin-responsive genes. Indeed, the expression of *SlCel4*, which was an auxin inducible gene (Brummell et al. 1997), and *SlXTH10*, which was highly expressed in the root (Saladié et al. 2006), increased in the NaCl-primed seeds/seedlings (Table 1), which is related to cell wall modification. Thus, it was suggested that the enhanced expression of the genes encoding enzymes involved in auxin signalling and cell wall modification promoted the growth of hypocotyl and root by NaCl-priming.

It has been reported that priming enables seeds to germinate more efficiently under unfavourable conditions, such as high or low temperatures (Rumpel and Szudyga 1978; Georghiou et al. 1982). The 300 mM NaCl-primed seeds germinated earlier than the non-primed and hydro-primed seeds under water stress conditions. This observation indicates not only that water uptake by seeds (hydro-priming) was a factor responsible for the acceleration of germination but also that NaCl itself might have played a role (Figure 5A). However, it has been reported that in some tomato cultivars grown under saline conditions, the fruit yield was higher in plants from primed seeds than in plants from non-primed seeds. This effect was more remarkable when the salt treatment was applied during germination than when it was applied at the seedling stage (Cano et al. 1991). It has also been reported that plants exhibit a higher capacity to adapt to salinity when salt treatments were applied during germination than when it was applied after emergence (Bolarín et al. 1993) and that melon seeds primed with NaCl for 3 d increased their salt tolerance during germination and the early stages of growth (Sivritepe et al. 2003).

Similarly, our results showed that under NaCl-stress conditions, the germination rate of 300 mM NaCl-primed seeds was significantly higher than that of the non-primed and hydro-primed seeds during the early germination stages (Figure 5B, C). With regard to the gene expression profile, NaCl-priming induces expression of *SlApx1* and *SlTFT7* genes (Table 1). It has been reported that up-regulation of *SlApx1* under salt stress might be related to protection against salt-induced oxidative stress (Najami et al. 2008), and *SlTFT7* promotes ascorbate peroxidase activity (Xu and Shi 2007). Thus, it was strongly suggested that the higher germination ratio under the salinity stress in the NaCl-

primed seed was caused by the modified gene expression and that this higher ratio would affect the subsequent adaptability to the salinity.

The 300 mM NaCl-primed plants exhibited a tolerance to bacterial wilt (Figure 6). In Arabidopsis, it was reported that resistance to Ralstonia solanacearum 1 (*RRS1*) genes and *RPS4* which provides resistance to *Pseudomonas syringae* pathovar *tomato* DC3000 expressing AvrRps4 compose a dual resistance gene system that prevents infection by three distinct pathogens including *R. solanacearum* (Narusaka et al. 2009). However, in this study, we could not determine a homologous gene to the resistance genes shown in Table 1 and S1. It has been reported that genes relative to both ethylene and salicylic acid defence signal transduction pathways were up-regulated in tomato plants infected by *R. solanacearum* (Milling et al. 2011). In addition, transgenic tomatoes overexpressing ethylene-responsive transcription factor 5 gene (*SlERF5*) displayed better tolerance to bacterial wilt (Li et al. 2011). Resistance to *R. solanacearum* in the tomato might be achieved, at least in part, by triggering the salicylic acid-defence-signalling pathway (Pan et al. 2010). In this study, the expression of PR-protein genes, 1-aminocyclopropane 1-carboxylate synthase 4 (*SlACS4*) and *tWRKY3*, which are induced by wounding and salicylic acid (Lincoln et al. 1993; Chen and Chen 2000), were enhanced (Table 1, S1). Our results suggest that the tolerance to bacterial wilt is acquired by the activation of these resistant genes during NaCl-priming.

Tolerance to biotic stress was maintained for at least 6 weeks (Figure 6). It was reported the primed plants display a faster and stronger activation of the various defence responses that are induced following attack by pathogens, insects and various abiotic stress (Conrath et al. 2006). A number of reports demonstrate that the effect of priming treatment on plants continues for at least several days. However, in the case of seed priming, the effect continues longer (Bruce et al. 2007). It has been reported that elevated sucrose synthase and glutamate synthase activities were observed in the seed-primed nodule of the chickpea, and these elevated activities might be responsible for increasing the nodule biomass and metabolic activity, thereby increasing seed fill (Kaur et al. 2006). Thus, the acquired tolerance to abiotic and biotic stress observed in the present study suggests that the effect of the seed-priming treatment continues longer and is more effective than the plant-priming treatment.

It has been reported that plant hormones participate in this process by inducing the expression of genes associated with stress tolerance (AbuQamar et al. 2009; Amitai-Zeigerson et al. 1995). The relationships among seed priming, stress tolerance and plant hormones still remain unclear in tomato. Therefore, in addition to seed germination, it is important to examine the changes in the plant hormone levels in the primed plant.

In conclusion, our results demonstrate that the low salt seed priming is effective in the promotion of germination and seedling emergence and to enhance the stress tolerance and subsequent growth of tomato plants. These processes are accompanied by modification of the expression of genes related to growth, abiotic and biotic stress. The established method will contribute to the production of high-quality tomato seedlings. Before applying the method to other crops, it will be important to elucidate the biochemical and molecular mechanisms underlying this phenomenon.

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