Induced Tolerance to Salinity Stress by Halotolerant Bacteria *Bacillus aryabhattai* H19-1 and *B. mesonae* H20-5 in Tomato Plants^S

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Salinity is one of the major abiotic stresses that cause reduction of plant growth and crop productivity. It has been reported that plant growth-promoting bacteria (PGPB) could confer abiotic stress tolerance to plants. In a previous study, we screened bacterial strains capable of enhancing plant health under abiotic stresses and identified these strains based on 16s rRNA sequencing analysis. In this study, we investigated the effects of two selected strains, *Bacillus aryabhattai* H19-1 and *B. mesonae* H20-5, on responses of tomato plants against salinity stress. As a result, they alleviated decrease in plant growth and chlorophyll content; only strain H19-1 increased carotenoid content compared to that in untreated plants under salinity stress. Strains H19-1 and H20-5 significantly decreased electrolyte leakage, whereas they increased Ca²⁺ content compared to that in the untreated control. Our results also indicated that H20-5-treated plants accumulated significantly higher levels of proline, abscisic acid (ABA), and antioxidant enzyme activities compared to untreated and H19-1-treated plants during salinity stress. Moreover, strain H20-5 upregulated *9-cisepoxycarotenoid dioxygenase 1* (*NCED1*) and *abscisic acid-response element-binding proteins 1* (*AREB1*) genes, otherwise strain H19-1 downregulated *AREB1* in tomato plants after the salinity challenge. These findings demonstrated that strains H19-1 and H20-5 induced ABA-independent and -dependent salinity tolerance, respectively, in tomato plants, therefore these strains can be used as effective bio-fertilizers for sustainable agriculture.

Keywords: *Bacillus aryabhattai*, *B. mesonae*, salinity stress, tomato, tolerance

Introduction

Several environmental factors, such as drought, salinity, water flooding, and extreme high and low temperature can affect plant growth and development; thus reducing crop productivity [1, 2]. Among these stresses, soil salinity is one of the most critical environmental stresses that constrains agricultural productivity [3]. It is known that 20% of all irrigated agricultural land as well as 10% of land surface and 50% of arable land are affected by soil salinity [4]. Furthermore, it is expected that 50% of the agricultural area will be salinized by 2050 [5]. Therefore, studies on strategies of crop management against salinity stress are essential.

Breeding of new cultivars and stress tolerant plants are studied as common crop management strategies against salinity stress [6]. Moreno-Limon *et al.* [7] and Mano and Takeda [8] developed salt tolerant cultivars of bean and wheat, respectively, with higher root growth and accumulation of mineral nutrients under salinity stress [9]. Moreover, genetic engineering approaches to improve tolerance to abiotic stress were attempted in rice and tomato by controlling plant signal transduction and regulation of biosynthesis and phytohormone pathway [10, 11]. However, these are long-term strategies, and many governments have not embraced them widely [12]. Therefore, use of microorganisms, including plant growth-promoting bacteria (PGPB) can be an alternative to avoid

the disadvantages of other strategies [13].

PGPB are well known as free-living soil bacteria that improve plant growth and productivity by increasing nutrient uptake and sustaining soil productivity [14]. Many studies have revealed that they can mitigate plant damage under abiotic stress conditions by the modulation of physiological, biochemical and molecular processes [15, 16, 17]. Many PGPB can produce 1-aminocyclopropane-1-carboxylate (ACC) deaminase which degrades the ethylene precursor ACC thereby alleviating damage by abiotic stress [18]. In addition, some PGPB can also produce indole-3-acetic acid (IAA) and affect phosphate solubilization activity. These bacterial traits play pivotal roles in plant growth promotion and mitigation of plant damage by enhancing nutrient absorption under salinity stress condition [19]. Moreover, they can regulate phytohormone synthesis through abscisic acid and antioxidant levels in plants, and alleviate osmotic stress-induced damage [20, 21]. Yang *et al.* [22] also proposed the term "Induced Systemic Tolerance (IST)", which is a PGPB-induced tolerance by regulating nutrient uptake, ion translocation, phytohormone and reactive oxygen species (ROS) against abiotic stresses in plants. Therefore, PGPB can be one of the possible strategies for alleviating damage by salinity stress. *Bacillus vallismortis* BS07M was also reported to promote plant growth and reduce soft rot in Chinese cabbages [23]; therefore, in this study, strain BS07M was used as a plant growth-promoting bacterium.

In our previous study, two bacterial strains, *Bacillus aryabhattai* H19-1 and *B. mesonae* H20-5, were selected from among 1,944 strains as bio-fertilizers for alleviation of growth reduction under salinity stress in tomato plants. In this study, we (i) investigated the mitigating effects of two pre-selected bacterial strains on plant growth, pigment and electrolyte leakage in leaves under salinity stress condition; (ii) demonstrated bacterial-mediated salinity tolerant mechanisms including ion content and osmolyte accumulation and (iii) examined bacteria-mediated regulation of ABA metabolism for enhancing stress tolerance in tomato plants. Through these studies, we attempted to understand the changes in plants and mitigating mechanisms of the two bacterial strains, H19-1 and H20-5, under salinity stress in tomato plants.

Materials and Methods

Plant Materials and Bacterial Treatments

Tomato (*Solanum lycopersicum* 'Superdotaerang') seeds were sown into plastic pots (diameter 9 cm) containing commercial

potting mixture (Bunong, Korea) and grown at room temperature in a greenhouse. Bacterial strains were cultured on tryptic soybean agar (TSA) at 28°C for 24 h, and a single colony was cultured in 200 ml of TSB at 28°C, 150 rpm for 48 h. The supernatant was removed after centrifugation at 6,000 ×g for 10 min, and cell suspension was adjusted to an OD₆₀₀ value of 0.25 with 10 mM MgSO₄. Bacterial suspensions (1 ml/g of potting mixture) were drenched into the pot-grown, three-week-old tomato seedlings. *B. vallismortis* BS07M (Bacillo V3, NaproBiotec Inc., Korea) was used as plant growth-promoting bacteria; 10 mM MgSO₄ was used as untreated control.

Challenging Salinity Stress

One week after bacterial treatment, balanced salinity solution (-1,000 kPa, *i.e.*, electrical conductivity (EC) 36.5 mS/cm, 1 ml/g of potting mixture) (as challenging salinity stress) was added to a pot three times at intervals of two days [24]. Non-stressed plants were irrigated only with distilled water (1 ml/g of potting mixture). Three weeks after challenging stress, plant growth and parameters including pigment content, electrolyte leakage, ion concentration and osmolyte content were evaluated.

Effect of Strains H19-1 and H20-5 on Growth of Tomato Plants

Preparation of tomato plants and challenging salinity were followed as described above. Three weeks after challenging salinity stress, plant growth including fresh weight (FW), dry weight (DW) and leaf pigment, such as chlorophyll and carotenoid, were measured. Plants (seven-week old, *i.e.*, ninth to tenth leaf stage) were taken out of soils in pots and roots were washed with running tap water. After removing soils and moisture, total fresh weight was measured. For dry weight, plants were dried at 60°C for three days and estimated. For measurement of chlorophyll and carotenoid content, sampled leaves were treated with 80% acetone (v/v) containing 0.5% (wt./v) MgCO₃ overnight at 4°C to prevent chlorophyll acidification. The crude extract was used to measure chlorophyll content according to the method of Lichtenthaler [25] using a UV/VIS spectrophotometer (Infinite M200 PRO, TECAN, Austria). Chlorophyll a, b, and carotenoid content were calculated as follows:

$$\text{Chlorophyll } a = (12.25 \times A_{663.2}) - (2.79 \times A_{646.8})$$

$$\text{Chlorophyll } b = (21.50 \times A_{646.8}) - (5.10 \times A_{663.2})$$

$$\text{Total chlorophyll content} = \text{chlorophyll } a + \text{chlorophyll } b$$

$$\text{Carotenoid concentration} = [(1,000 \times A_{470}) - (1.8 \times \text{chlorophyll } a) - (85.02 \times \text{chlorophyll } b)]/198$$

Changes in Electrolyte Leakage, Ion Concentration, Free Proline, Total Soluble Sugar and Free Amino Acid

For measurement of electrolyte leakage, a method of Lutts *et al.* [26] was used with modifications by Shukla *et al.* [27]. Leaves were cut and washed with deionized water to remove electrolytes

adhering to the leaf surface. The washed leaves were transferred into test tubes containing 10 ml of deionized water, and the leaves were incubated at 25°C for 24 h. Electrical conductivity (EC) of the solution (L_t) was measured, and the samples were autoclaved at 121°C for 20 min. After cooling at room temperature, the final EC (L_0) was detected. Electrolyte leakage (%) was calculated as follows:

$$\text{Electrolyte leakage} = (L_t/L_0) \times 100$$

For measurement of ion content in tomato leaves, Na^+ , K^+ , and Ca^{2+} ion meters (LAQUAtwin, Japan) were used as described by Iseki *et al.* [28]. Similar-sized plant leaves (50–65 mg) were sampled and dried at 70°C for three days. The dried leaves were weighed and homogenized using Retsch MM200 Mixer (RetshTM, Germany). After adding 1 ml of distilled water, the samples were incubated at 150 rpm for 1 h, spun down at 7,000 $\times g$ for 5 min, and the supernatant was used for measuring ion content.

Free proline content in plant leaves was estimated by a ninhydrin test [29]. Plant leaves (100 mg) were homogenized in 1.2 ml of 3% sulfosalicylic acid, and samples were centrifuged at 13,000 $\times g$ for 10 min. Supernatant 500 μl was mixed with 500 μl of glacial acetic acid and 500 μl of acidic ninhydrin, and then the mixture was heated at 90°C for 1 h. The samples were cooled in ice and 1 ml of toluene was added. After vortexing, the upper phase showed absorbance at 520 nm.

For measurement of total soluble sugar and free amino acid, leaf (1 g) was extracted with methanol-chloroform-water (60:25:15 v/v) at 60°C for 2 h. After centrifugation at 10,000 $\times g$ for 10 min, the supernatant was used to estimate total soluble sugar content by anthrone-sulfuric acid method [30]. Total free amino acid content was measured according to the method of Chen *et al.* [31] using a ninhydrin test. Next, 0.1 M sodium acetate acetic buffer (pH = 4.3, 1 ml) and 5% ninhydrin agent (1 ml) were added in the plant extract (1 ml) and the mixture was heated immediately at 95°C for 15 min. The samples were cooled at room temperature and absorbance was read at 570 nm.

Antioxidant Enzyme Activities and ABA Accumulation

Enzyme activities of catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) were determined six days after challenging stress. For measurement of CAT activity, 1 g of frozen leaves were homogenized in 5 ml of cold 50 mM potassium phosphate buffer (pH = 7.0) containing 1 mM EDTA and then centrifuged at 12,000 $\times g$ for 15 min at 4°C. CAT activity was measured in the supernatant with a catalase assay kit (Item no. 707002, Cayman Chemical Company, USA). For measurement of SOD activity, 1 g of frozen leaves were homogenized in 5 ml of cold 20 mM HEPES buffer (pH = 7.2) containing 1 mM EGTA, 210 mM mannitol and 70 mM sucrose. After centrifuging at 12,000 $\times g$ for 15 min at 4°C, supernatant was used to estimate SOD enzyme activity by using a superoxide dismutase assay kit (Item no. 706002, Cayman Chemical Company). GPx activity was measured using glutathione peroxidase assay kit (item no. 703102, Cayman Chemical Company). One milliliter of homogenization

buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA and 1 mM DTT) was added per 100 mg of frozen leaves and homogenized. After spinning down at 12,000 $\times g$ for 15 min at 4°C, supernatant was used. ABA content in plant leaves was tested by the enzyme-linked immunosorbent assay (ELISA) with an ABA test kit (Cat. No. PDK 09347/0096, Agdia Inc., USA) following the manufacturer's instructions and methodology described by Iovieno *et al.* [32].

Gene Expression Analysis

Tomato leaves were collected and frozen by liquid nitrogen at 6, 24 and 48 h after challenging stress. The tissues were homogenized using a Retsch MM200 Mixer Model Mill (RetshTM) and total RNA was extracted using a plant RNA extraction kit (iNtRON, Korea) according to the manufacturer's instructions. For the quantitative reverse transcription polymerase chain reaction (qRT-PCR), complementary DNA (cDNA) was synthesized using the PrimeScript RT Master Mix (Cat. No. RR036A, Takara). qRT-PCR analysis performed with 20 μl of reaction mixture containing 3 μl cDNA (10% of the first-strand reaction), 1 μl of each primer (5 pmol), and 2 $\times$ SYBR reaction buffer (TOPreadTM qPCR 2 $\times$ PreMIX, Enzynomics, Korea). Amplification was carried out using a CFX96 Real-Time PCR Detection System (Bio-Rad, USA) and an initial denaturation step was performed at 95°C for 10 min, followed by 40 cycles for 15 sec at 95°C, 20 sec at 58°C, and 18 sec at 72°C. For quantification of relative gene expression, primer sequences of the genes related to ABA-dependent pathway (*9-cis-epoxycarotenoid dioxygenase 1* (NCED1) and *abscisic acid-response element-binding proteins* (AREB1)) are listed in Table S1. Actin was utilized as reference gene described by Løvdaal and Lillo [33] and the relative gene expression levels were evaluated using $2^{-\Delta\Delta\text{Ct}}$ method [34].

Statistical Analysis

Statistical analysis of the data obtained was performed using Statistical Analysis System (SAS) (version 9.1.3, SAS Institute Inc., Cary, NC). Plant growth, leaf pigment, electrolyte leakage, ion content and osmolyte tests were conducted with 12 replications from two experiments; for antioxidant enzyme activities, ABA accumulation, and gene expression analysis eight replications were conducted from two experiments. Pooled data from repeated experiments were used for statistical analysis after confirmation of homogeneity of the variances by Leven's test. Analysis of variance was performed using the general linear model (GLM), and the means were separated by the least significant difference (LSD), or Tukey's tests at $p < 0.05$.

Results

Strains H19-1 and H20-5 Promote Plant Growth and Leaf Pigment Content under Salinity Stress

Three weeks after the salinity stress challenge in tomato plants, growth inhibition was observed in stressed plants

Fig. 1. The effects of *B. aryabhattai* H19-1 and *B. mesonae* H20-5 on (A) growth, (B) fresh weight, and (C) dry weight in tomato plants under non- and salinity stresses.

Bacterial suspensions and 10 mM MgSO_4 (untreated) were drenched into tomato plants grown for three weeks in a greenhouse. One week after treatments, water (for non-stress) or salinity solutions (for stress) were drenched into plants; three weeks after salinity stress challenge, plant growth was measured. Asterisks on the bar mean statistical difference by the LSD test at $p < 0.05$; error bars indicate standard error.

compared to non-stressed (Fig. 1, fresh weight (FW): $p < 0.0001$; dry weight (DW): $p < 0.0001$). In non-stressed plants, fresh weight was not significantly different between untreated and bacteria-treated plants (Fig. 1B, $p = 0.8124$). However, compared to plants under salinity stress, both

bacterial strains, H19-1 and H20-5 led to an increase of 20% and 19%, respectively, in fresh weight ($p = 0.0014$). Similarly, the dry weight of plants treated with the two bacterial strains, H19-1 and H20-5, was 25% and 21%, respectively, higher than that of untreated plants under salinity stress,

Table 1. Total chlorophyll and carotenoid content in leaves of tomato plants treated with 10 mM MgSO_4 (untreated) or bacterial suspensions under non- and salinity stresses.

Treatment	Chlorophyll content ($\mu\text{g/g FW}$)		Carotenoid content ($\mu\text{g/g FW}$)	
	Non-stress	Salinity stress	Non-stress	Salinity stress
Untreated	137.5 $\pm$ 3.4 ^a	118.7 $\pm$ 1.6	29.5 $\pm$ 0.7	23.0 $\pm$ 0.8
BS07M	135.8 $\pm$ 5.8	113.4 $\pm$ 2.8	28.8 $\pm$ 1.0	23.2 $\pm$ 0.5
H19-1	153.2 $\pm$ 7.1*	131.1 $\pm$ 2.0*	34.1 $\pm$ 2.1*	26.4 $\pm$ 0.4*
H20-5	146.0 $\pm$ 2.8	127.0 $\pm$ 3.7*	33.0 $\pm$ 1.7	23.5 $\pm$ 0.9

^aThe values are mean $\pm$ standard errors of 12 replications from two experiments. An asterisk indicates a statistical difference ($p < 0.05$) compared to untreated plants in a column by the LSD test.

whereas no significant difference was observed in non-stressed plants (Fig. 1C, non-stressed plants: $p = 0.7493$; salinity-stressed plants: $p = 0.0013$). Photosynthetic pigments containing chlorophyll and carotenoid content were decreased in stressed plants compared to that in non-stressed plants (Table 1, chlorophyll content: $p < 0.0001$; carotenoid content: $p < 0.0001$). The pigment content of plants treated with H19-1 was higher than that of untreated plants under non-stressed condition (chlorophyll content: $p = 0.0455$; carotenoid content: $p = 0.0377$). Moreover, significant increase of chlorophyll content was observed in plants treated by both strains under salinity stress while only H19-1 significantly elevated carotenoid content in stressed plants (chlorophyll content: $p = 0.0008$; carotenoid content: $p = 0.0078$).

Strains H19-1 and H20-5 Decrease Electrolyte Leakage and Increase Ca^{2+} Content under Salinity Stress

To investigate the influence of bacterial treatment on plant biochemical properties under salinity stress, electrolyte leakage, and high ion concentrations including those of Na^+ , K^+ , and Ca^{2+} were evaluated in plant leaves. Our present study indicated that salinity stress led to an increase in electrolyte leakage in plant leaves compared to non-stressed plants (Fig. 2, $p < 0.0001$). Moreover, treatment of H19-1 and H20-5 decreased electrolyte leakage in plants by 15% and 17%, while untreated plants showed 36%

Fig. 2. The effects of *B. aryabhattai* H19-1 and *B. mesonae* H20-5 on electrolyte leakage (%) in tomato leaves under non- and salinity stresses.

Leaves were sampled at three weeks after the salinity stress challenge. Asterisks on the bar mean statistical difference by the LSD test at $p < 0.05$ and error bars indicate standard error.

electrolyte leakage under salinity stress ($p = 0.0073$). Under non-stress condition, no evident difference between untreated and bacteria-treated plants was observed ($p = 0.2564$). Concentration of Na^+ in non-stressed and stressed

Fig. 3. The effects of *B. aryabhattai* H19-1 and *B. mesonae* H20-5 on (A) Na^+ , (B) K^+ , and (C) Ca^{2+} contents (mmol/g DW) in tomato leaves under non- and salinity stresses.

Leaves were sampled at three weeks after salinity stress challenge. Asterisks on the bar mean significant difference by the LSD test at $p < 0.05$; error bars indicate standard error.

plants did not show any evident difference, whereas stress challenge led to an increase in K^+ concentration (Na^+ content: $p = 0.6243$; K^+ content: $p = 0.0001$). In addition, there was no significant difference in these concentrations between untreated and bacteria-treated plants under both conditions (Figs. 3A and 3B, Na^+ (non-stressed plants): $p = 0.6298$; Na^+ (salinity-stressed plants): $p = 0.2731$; K^+ (non-stressed plants): $p = 0.0802$; K^+ (salinity-stressed plants): $p = 0.4355$). In contrast to Na^+ and K^+ concentration, salinity stress led to a decrease in Ca^{2+} concentration in plant leaves compared to that in non-stressed plants ($p < 0.0001$). Moreover, Ca^{2+} concentrations in tomato plants treated with H19-1 and H20-5 were 24% and 35% higher than that in untreated plants in salinity-stressed plants but not in non-stressed plants (Fig. 3C, non-stressed plants: $p = 0.7346$; salinity-stressed plants: $p = 0.0003$).

Strain H20-5 Accumulates Proline in Tomato Plants but Did Not Affect Total Soluble Sugar and Free Amino Acid under Salinity Stress

To demonstrate the effect of bacterial strains on osmoprotection in plants, contents of proline, total soluble sugar and free amino acid were measured. As a result, stress challenge led to proline accumulation in plants compared to non-stressed plants (Table 2, $p < 0.0001$). Moreover, proline content was significantly accumulated in plants treated with H20-5 compared to that in untreated plants under salinity stress, whereas strains BS07M and H19-1 did not affect proline content in plants ($p = 0.0410$). In non-stressed condition, changes in proline content were not observed ($p = 0.6345$). Total soluble sugar and free amino acid contents in plants were also decreased under salinity stress (total soluble sugar: $p < 0.0001$; free amino acid: $p < 0.0001$). However, there was no significant difference between untreated and bacteria-treated plants in both non-stressed and stressed conditions (total soluble sugar (non-stressed plants): $p = 0.8565$; total soluble sugar (stressed plants): $p = 0.8251$; free amino acid (non-stressed plants): $p = 0.6303$; free amino acid (stressed plants): $p = 0.7574$).

Strain H20-5 Enhances Antioxidant Enzyme Activities during Salinity Stress

To examine the mitigative effect of bacterial treatment on oxidative damage in plants, antioxidant enzymes activities were evaluated. Three antioxidant enzyme activities were increased in plants in salinity stress conditions, compared to those in non-stressed conditions (Fig. 4, catalase (CAT): $p < 0.0001$; superoxide dismutase (SOD): $p < 0.0001$;

Fig. 4. The effects of *B. aryabhattai* H19-1 and *B. mesonae* H20-5 on antioxidant enzyme activities including (A) CAT, (B) SOD, and (C) GPx in tomato leaves under non- and salinity stresses. Leaves were sampled at six days after salinity stress challenge. Asterisks on the bar mean significant difference by the LSD test at $p < 0.05$; error bars indicate standard error.

glutathione peroxidase (GPx): $p = 0.0003$). Antioxidant enzyme activity assays showed no significant difference

Table 2. Proline, total soluble sugar and free amino acid contents in leaves of tomato plants treated with 10 mM MgSO₄ (untreated) or bacterial suspensions under non- and salinity stresses.

Treatment	Proline (mg/g DW)		Total soluble sugar (mg/g DW)		Total free amino acid (μmol/g DW)	
	Non-stress	Salinity stress	Non-stress	Salinity stress	Non-stress	Salinity stress
Untreated	0.62 ± 0.19 ^a	59.85 ± 2.08	9.50 ± 0.53	4.36 ± 0.29	101.88 ± 2.58	61.20 ± 1.14
BS07M	0.83 ± 0.19	61.05 ± 3.31	9.64 ± 0.35	4.59 ± 0.25	101.22 ± 6.24	59.93 ± 2.18
H19-1	0.79 ± 0.18	56.45 ± 2.91	9.84 ± 0.20	4.42 ± 0.31	106.52 ± 3.19	60.51 ± 2.50
H20-5	0.96 ± 0.20	69.15 ± 3.43*	9.96 ± 0.30	4.70 ± 0.22	109.70 ± 6.22	58.40 ± 1.66

^aThe values are mean ± standard errors of 12 replications from two experiments. An asterisk indicates a statistical difference ($p < 0.05$) compared to untreated plants in a column by the LSD test.

between treatments under non-stressed condition (CAT: $p = 0.4640$; SOD: $p = 0.9147$; GPx: $p = 0.1599$). However, H20-5 treatments significantly increased the activities of CAT (Fig. 4A), SOD (Fig. 4B) and GPx (Fig. 4C) compared to untreated plants under stressed condition whereas H19-1 did not (CAT: $p = 0.0116$; SOD: $p = 0.0206$; GPx: $p = 0.0253$).

Strain H20-5 Regulates ABA Accumulation and ABA-Related Gene Expression in Tomato Plants under Salinity Stress

To examine the effect of bacterial treatment on ABA metabolism in plants under salinity stress, ABA content and the expression levels of ABA-related genes including *NCED1* and *AREB1* were analyzed at 0, 6, and 24 h after stress challenge in plant leaves. H20-5-treated plants accumulated significantly higher levels of ABA than that in untreated and H19-1-treated plants at 24 h after salinity stress challenge. (Fig. 5, 0 HAS: $p = 0.9679$; 6 HAS: $p = 0.3495$; 24 HAS: $p = 0.0055$). Interestingly, bacterial strain H19-1 did not affect ABA content in leaves of tomato plants

under salinity stress. In contrast to stressed plants, no striking difference was observed between untreated and bacteria-treated plants in the non-stressed condition (0 h after stress challenge (HAS): $p = 0.2267$; 6 HAS: $p = 0.5718$; 24 HAS: $p = 0.2220$). Relative gene expression of *NCED1* and *AREB1* had no significant difference between untreated and treated plants under non-stressed condition (Fig. 6, 0 HAS (*NCED1*): $p = 0.1396$; 6 HAS (*NCED1*): $p = 0.0049$; 24 HAS (*NCED1*): $p = 0.3382$; 0 HAS (*AREB1*): $p = 0.1531$; 6 HAS (*AREB1*): $p = 0.0117$; 24 HAS (*AREB1*): $p = 0.7169$). In stressed conditions, *NCED1* gene expression level was increased in leaves of tomato plants treated with H20-5 at 6 h after stress challenge (Fig. 6A, $p = 0.0168$); *AREB1* gene expression was also seen to increase in leaves of plants treated with H20-5 at 6 and 24 h after the stress challenge (Fig. 6B, 6 HAS: $p = 0.0001$; 24 HAS: $p < 0.0001$). However, compared to untreated plants, H19-1 treatment led to a decrease in *AREB1* gene expression level in plants at 6 and 24 h after stress challenge.

Fig. 5. The effects of *B. aryabhattai* H19-1 and *B. mesonae* H20-5 on ABA accumulation in tomato leaves under non- and salinity stresses.

Leaves were sampled at 0, 6, and 24 h after salinity stress challenge. Asterisks on the bar mean significant difference by the LSD test at $p < 0.05$; error bars indicate standard error.

Fig. 6. The effects of *B. aryabhattai* H19-1 and *B. mesonae* H20-5 on gene expression level of (A) *NCED1* and (B) *AREB1* in tomato leaves under non- and salinity stresses.

Leaves were sampled at 0, 6, and 24 h after drenching water or salinity solution. Asterisks on the bar mean significant difference by the Tukey's test at $p < 0.05$; error bars on the bar indicate standard error.

Discussion

To alleviate salinity stress in plants, beneficial bacterial strains such as PGPB and endophytic bacteria have been used to regulate plant abiotic stress tolerance [35, 36]. In our previous study, two halotolerant bacterial strains, *B. aryabhattai* H19-1 and *B. mesonae* H20-5, were selected [37]. In this study, we demonstrated the changes in pigment content, electrolyte leakage, and Ca^{2+} content in tomato plants under salinity stress. Furthermore, we revealed that strain H20-5 regulated proline accumulation, antioxidant enzyme activities and ABA related metabolism in stressed plants.

Salinity stress decreases crop development and yield by down regulating photosynthesis including chlorophyll biosynthesis [38, 39]. Carotenoid can also regulate plant photosynthesis by helping in harvesting light and photoprotection [40, 41]. In our study, fresh and dry weight

as well as chlorophyll content in plants treated with H19-1 and H20-5 were increased compared to that in the control, whereas carotenoid content was increased in plants treated with H19-1 under salinity stress. These results indicated that the two bacterial strains regulate photosynthesis by increasing chlorophyll content; strain H19-1 protects carotenoid-related photosynthesis in plants under salinity stress. Similarly, earlier reports revealed that *Sinorhizobium meliloti* and *Paenibacillus yonginensis* help increase chlorophyll and carotenoid content under salinity stress in lucerne and ginseng plants, respectively [42, 43].

Electrolyte leakage is involved in programmed cell death and metabolic adjustment of plants under stresses such as salinity, drought, and pathogen attack; therefore, it was used as a parameter for assessing cell membrane stability [44, 45]. Both strains H19-1 and H20-5 decreased leaf electrolyte leakage compared to that in untreated plants under salinity stress. This result indicated the two bacterial strains help protect plants from cell membrane damage and enables them to adjust to salinity stress by regulating cell membrane function. Similarly, *Serratia liquefaciens*, *Pseudomonas* and *Klebsiella* sp. were reported to improve salinity stress tolerance by decreasing electrolyte leakage in plants [46–48]. Therefore, strains H19-1 and H20-5 enhance tolerance to salinity stress by decreasing electrolyte leakage in leaves of tomato plants.

Extreme Na^+ accumulation in plants can limit absorption, transport, and distribution of diverse mineral nutrients, and lead to nutrient imbalance in plants, e.g. decrease in K^+ and Ca^{2+} content [49, 50]. Moreover, accumulation of Na^+ ion by root uptake induces Na^+ toxicity in the cytosol and disturbs the balance of Na^+ concentration in the vacuole of plants [51]. However, the two bacterial strains, H19-1 and H20-5, did not affect either Na^+ or K^+ content in non-stressed and stressed plants. This result could be caused by salinity solution components used in our study that comprised high concentrations of KNO_3 , $\text{Ca}(\text{NO}_3)_2$, MgSO_4 , and KH_2PO_4 ; therefore, accumulation of Na^+ did not occur in our systems and these may also not affect K^+ content in tomato leaves. Ca^{2+} content was decreased in plants under salinity stress, however, H19-1 and H20-5 accumulated higher levels of Ca^{2+} in leaves compared to those in untreated plants. Intracellular Ca^{2+} acts as a second messenger in stress perception and plays an important role in response to salinity stress by modulating the SOS signal transduction cascade which regulates ion homeostasis [52, 53]. In addition, Ca^{2+} accumulation was involved in osmoregulation ability by increasing proline and improving the efficiency of water

uptake under abiotic stress conditions [1, 54]. As a result, strains H19-1 and H20-5 induced salinity tolerance in tomato plants by Ca^{2+} accumulation.

Salinity stress has been reported to encourage proline accumulation for cell osmotic adjustment and membrane stabilization by regulating proline biosynthesis in plants [55]. Proline contributes to better chlorophyll protection, increase in water use efficiency, and protection of antioxidant enzymes [56]. In present study, only strain H20-5-treated plants exhibited a higher level of proline content than that in untreated plants. Similarly, *Enterobacter cloacae* strain increased proline content in canola plants under salinity stress [57]. Strains *Bacillus subtilis* and *Arthrobacter* sp. also confer salinity tolerance in wheat plants by increasing leaf proline content [58]. Therefore, accumulation of proline by H20-5 is one of the induced tolerance mechanisms in tomato plants under our salinity stress system. Total soluble sugar and free amino acids in plants also play an important role in osmoprotection and adjustment to salinity stress by conferring desiccation resistance to plant cells, and thus, help prevent plant growth inhibition in stressed conditions [59, 60]. However, total soluble sugar and free amino acid did not accumulate under salinity stress in plants treated with bacterial strains. This result might indicate that induced salinity tolerance by strains H19-1 and H20-5 was not related to accumulation of total soluble sugars and free amino acids among a variety of plant osmolytes in our salinity system.

Plants have antioxidant defense systems including enzymes, which scavenge ROS in plant cells [61]. Salinity leads to overproduction of ROS including superoxide, hydroxyl ion, and singlet oxygen that cause damage to the membrane lipid and protein and the structure of the cell by membrane oxidation and thereby disrupt membrane semi-permeability and water uptake mechanisms [62, 63]. It is known that SOD, one of the antioxidant enzymes, induces the dismutation of superoxide to oxygen and hydrogen peroxide and CAT and GPx coordinated with SOD play a significant role in scavenging hydrogen peroxide, thereby providing protection, and leading to salinity stress tolerance [64]. Strain H20-5 improved SOD, CAT, and GPx activities under salinity stress whereas no significant effect was observed in non-stressed plants. Recently, similar results were reported, where beneficial microbes induced salinity tolerance by improving antioxidant enzyme activities in plants [65, 66]. Accordingly, H20-5 augments antioxidant enzyme activities and activation of the ROS scavenging process for enhancing tolerance to salinity stress in tomato plants.

ABA is well known as one of the phytohormones that is involved in signal pathways against abiotic stress. When plants encounter salinity stress, ABA in plants regulates the vacuolar Na^+/H^+ antiporter gene related to ion homeostasis in cells and stress-response genes, such as *RD29A*, *RAB18* and *P5CS* [67, 68]. Furthermore, ABA has been demonstrated to modulate transpiration in plants by increasing water influx into roots and controlling stomatal closure in leaves to adjust abiotic stress response [69, 70]. Recently, *Bacillus licheniformis*, *Azospirillum* sp. and *Pseudomonas* sp. were reported to induce salinity stress tolerance in plants through enhancement of ABA content in leaves [71, 72]. Similarly, only strain H20-5 increased ABA content compared to that in untreated plants at 24 h after the salinity stress challenge. This might mean H20-5 conferred induced-tolerance to salinity stress by inducing ABA accumulation in plants. Furthermore, relative expression level of the *NCED1* gene in plants treated with H20-5 was higher than that in untreated plants under salinity stress at 6 h after the salinity stress challenge. The enzyme 9-cisepoxycarotenoid dioxygenase (NCED) is key in the biosynthesis of ABA in plants [73]; the NCED encoded gene induced by abiotic stresses, led to ABA accumulation; overexpression of the *NCED* gene caused over-production of ABA, and enhanced abiotic stress tolerance [74, 75]. The abscisic acid-response element-binding proteins (AREBs), bZIP transcriptional factors, are known to mediate the gene activation pathway related to abiotic stress tolerance by recognition of ABA hormones in plants [76, 77]. Hsieh *et al.* [78] and Orellana *et al.* [79] have reported that *AREB1* is involved in ABA signals and confers salinity and drought stress tolerance by regulating ABA-dependent stress response genes. Interestingly, H20-5 increased *AREB1* gene expression level whereas H19-1 decreased it in tomato plants at 6 and 24 h after the salinity stress challenge. These results indicated that H20-5 led to upregulation of *AREB1* gene expression to confer salinity stress tolerance, whereas H19-1 caused downregulation of the same, presumably facilitating adaptation to the stressed condition. Accordingly, H20-5 regulated ABA metabolism including ABA accumulation and expression of ABA-related genes, *NCED1* and *AREB1* to tolerate salinity stress in tomato plants.

Briefly, we demonstrated the effects of bacterial strains *Bacillus aryabhattai* H19-1 and *B. mesonae* H20-5 on induced tolerance to salinity stress in tomato plants (Fig. 7). The findings revealed that H19-1 and H20-5 could ameliorate salinity stress damage in plants by inducing ABA-independent and -dependent tolerance, respectively. We

Fig. 7. Overview of mode of action of *B. aryabhattai* H19-1 and *B. mesonae* H20-5 for induced-tolerance to salinity stress in tomato plants.

The two bacterial strains improve plant growth, chlorophyll and Ca²⁺ ion accumulation and reduce electrolyte leakage in plant leaves. In addition, *B. mesonae* H20-5 lead to an increase of antioxidant enzyme activities including CAT, SOD, and GPx; ABA accumulation; upregulate two genes, *NCED1* and *AREB1* in tomato plants. On the contrary, *B. aryabhattai* H19-1 downregulate *AREB1* gene expression. The red and blue colored arrows indicate the increase and decrease of tested factors, respectively, in tomato plants.

propose that these two strains can be used as effective bio-fertilizer for sustainable agriculture by enhancement of plant tolerance to salinity stress.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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