Mol. Cells 2014; 37(2): 109-117
http://dx.doi.org/10.14348/molcells.2014.2239

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Alleviation of Salt Stress by *Enterobacter* sp. EJ01 in Tomato and *Arabidopsis* Is Accompanied by Up-Regulation of Conserved Salinity Responsive Factors in Plants

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Microbiota in the niches of the rhizosphere zones can affect plant growth and responses to environmental stress conditions *via* mutualistic interactions with host plants. Specifically, some beneficial bacteria, collectively referred to as Plant Growth Promoting Rhizobacteria (PGPRs), increase plant biomass and innate immunity potential. Here, we report that *Enterobacter* sp. EJ01, a bacterium isolated from sea china pink (*Dianthus japonicus thunb*) in reclaimed land of Gyehwa-do in Korea, improved the vegetative growth and alleviated salt stress in tomato and *Arabidopsis*. EJ01 was capable of producing 1-aminocy-clopropane-1-carboxylate (ACC) deaminase and also exhibited indole-3-acetic acid (IAA) production. The isolate EJ01 conferred increases in fresh weight, dry weight, and plant height of tomato and *Arabidopsis* under both normal and high salinity conditions. At the molecular level, short-term treatment with EJ01 increased the expression of salt stress responsive genes such as *DREB2b*, *RD29A*, *RD29B,* and *RAB18* in *Arabidopsis*. The expression of proline biosynthetic genes (i.e. *P5CS1* and *P5CS2*) and of genes related to priming processes (i.e. *MPK3* and *MPK6*) were also up-regulated. In addition, reactive oxygen species scavenging activities were enhanced in tomatoes treated with EJ01 in stressed conditions. GFP-tagged EJ01 displayed colonization in the rhizosphere and endosphere in the roots of *Arabidopsis*. In conclusion, the newly isolated *Enterobacter* sp. EJ01 is a likely PGPR and alleviates salt stress in host plants through multiple mechanisms, including the rapid up-regulation of conserved plant salt stress responsive signaling pathways.

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Received 30 August, 2013; revised 16 December, 2013; accepted 18 December, 2013; published online 19 February, 2014

Keywords: *Arabidopsis*, *Enterobacter*, PGPR, ROS, salt stress

INTRODUCTION

Soil salinity adversely affects plant growth and has become one of the major limiting factors in crop productivity worldwide. Mechanistically, high salinity primarily disrupts the cellular osmotic balance by lowering the water potential inside cells in a similar fashion to stresses induced by drought and freezing (Krasensky and Jonak, 2012). Prolonged salt stresses additionally cause ion toxicity due to increased concentrations of Na^+ and Cl^- ions. Such unfavorable conditions subsequently induce oxidative stresses by generating reactive oxygen species (ROS) such as superoxide, hydrogen peroxide, singlet oxygen, and hydroxyl radicals, which are detrimental to cell viability.

However, plants have adapted to salt stresses *via* conserved signal transduction pathways that activate various stress avoidance and tolerance mechanisms (Xiong et al., 2002). In these processes, a phytohormone Abscisic acid (ABA) is known to play major roles *via* modulating the expression of genes that possess ABA-responsive elements (ABREs) in the promoter regions. Another *cis*-acting element known as dehydration-responsive element (DRE) also plays an important role in salt stress responses in plants. In the case of *Arabidopsis*, *RD29A* and *RD29B* are representative regulons of ABRE and DRE, and their transcription is modulated by upstream transcription factors, DRE-binding proteins (DREB)-like genes. As a result of such signaling, diverse functional components like Late Embryogenesis Abundant (LEA) genes (e.g., *RAB18*), osmolytes, antioxidants, and reactive oxygen species (ROS) scavengers are activated and mitigate the oxidative damage caused by salinity (Lata and Prasad, 2011; Xiong et al., 2002; Zhu, 2002).

Various bacteria collectively referred to as Plant Growth Promoting Rhizobacteria (PGPRs) colonize the rhizosphere of many plant species and have beneficial effects on host plant growth, which is manifested physiologically as increased plant growth and reduced susceptibility to diseases (Bulgarelli et al., 2013). Many PGPRs exhibit properties of phosphorus solubili zation and siderophore production, which allow host plants to efficiently uptake phosphorus- and iron-derived nutrients from the soil, respectively. In addition, PGPRs often interfere with the biosynthesis of phytohormones, especially auxin and ethylene. Auxins are majorly produced from indole-3-acetamide (IAM)

eISSN: 0219-1032
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and indole-3-pyruvate (IPyA), precursors excreted from many soil- and plant-associated bacteria (Dodd and Perez-Alfocea, 2012; Spaepen and Vanderleyden, 2011). The direct linkage between bacterial indole acetic acid (IAA) production and modified root architecture of the host was clearly demonstrated between *A. brasilense* and wheat (Spaepen et al., 2007). However, IAA biosynthesis *per se* did not account for the beneficial effects of PGPR, because IAA knockout mutants still promoted plant growth (Spaepen and Vanderleyden, 2013). On the other hand, the level of ethylene, a phytohormone involved in senescence and growth inhibition of plants (Bulgarelli et al., 2013), has also been considered as a major indicator of PGPR effects on host plants. Many microbes excrete 1-aminocyclopropane-1-carboxylate deaminase (ACCD), which is encoded by the conserved *acdS* gene, and degrade the ethylene precursor ACC to ammonia and α-ketobutyrate, thereby reducing the level of ethylene. In addition, volatile compounds (VOCs) such as 3-hydroxy-2-butanone (acetoin) and 2,3-butanediol generated by microbes have also been suggested to modulate host growth (Ryu et al., 2003). As mentioned, the modes of action of PGPR-related factors are thus diverse and complex, but the mechanistic basis is still largely unknown, especially at the molecular level.

Several PGPRs have also been reported to enhance the drought and salt stress resistance of their hosts (Dodd and Perez-Alfocea, 2012; Yang et al., 2009). For example, *Achromobacter piechaudii* ARV8 producing ACCD conferred tolerance to drought and salt stresses in peppers and tomato (Mayak et al., 2004a; 2004b). *Arabidopsis* treated with *Bacillus subtilis* GB03 also showed enhanced salt tolerance (Zhang et al., 2008). In recent reports, combinatorial treatments of multiple microbes boosted salt tolerance in mung bean, tomato, and brahmi (Ahmad et al., 2011; Bharti et al., 2013; Shen et al., 2012). At the mechanistic level, such tolerance is largely explained by means of water homeostatsis, osmolyte accumulation, antioxidants, hormone homeostasis, the production of ACCD and volatile compounds, and sodium uptake/transport (Dodd and Perez-Alfocea, 2012; Yang et al., 2009). Altered levels of the stress phytohormone ABA were mainly attributed to stress resistance (Aroca et al., 2006; Jahromi et al., 2008). PGPRs isolated from halophytic weeds produce ABA *in vitro* and enhanced salt tolerance in soybean (Naz et al., 2009). The production of ACCD was also suggested to be linked to drought tolerance, because abiotic stresses concomitantly increased the level of ethylene by up-regulating biosynthetic genes (Achard et al., 2006; Cao et al., 2006). Regardless of reports suggesting the positive effects of PGPRs in the saline rhizosphere, however, the modes of microbial action and their phyto-effects are multifarious and have yet to be delineated in detail.

In this study, we aimed to isolate a bacterium beneficial to plant growth and salt stress resistance, and investigated the effects of a PGPR on the expressions of conserved stress-responsive genes. *Enterobacter* sp. EJ01 facilitated plant growth and increased the salt tolerance of *Arabidopsis* and tomato *via* colonization of the host root tissues. The mechanisms underlying the acquired salt tolerance were apparently complex and likely included multiple physiological processes. Bacterial treatment rapidly induced the post-transcriptional modulation of salt stress responses of the hosts. Our data suggest that EJ01-driven salt tolerance includes the rapid activation of conserved salt stress-responsive signaling pathways in host plants.

MATERIALS AND METHODS

Plant materials and growth conditions

Arabidopsis thaliana ecotype Columbia (Col-0) and tomato (*Lycopersicon esculentum var.* Mill) were used in this study. Seeds were surface sterilized with 2% sodium hypochlorite for 5 min, followed by rinsing with sterile water. For germination of *Arabidopsis*, seeds were put on media containing half-strength MS media (Duchefa, Japan) and 0.5% sucrose under fluorescent light (15 h light / 9 h dark) at 22°C. Then, ten-day-old *Arabidopsis* seedlings were transferred to plastic pots ($5 \times 5 \times 5$ cm) filled with perlite and vermiculite (1:1) and grown further in soil under the same light regime. For tomato, seeds were germinated in wet filter paper for five days. Seedlings were then transferred to plastic pots ($7 \times 7 \times 7$ cm) filled with horticultural soil mix (cocopeat 60%, peat moss 20%, vermiculite 10%, perlite 7%, seaweed mix 3%) and grown under the same conditions described for *Arabidopsis.*

To monitor plant growth and salt tolerance, two days after transfer to pots, *Arabidopsis* and tomato seedlings were inoculated with bacterial suspensions prepared as follows. *Enterobacter* sp. EJ01, the bacterium isolated in the current study, was cultured in LB broth overnight at 30°C, and cell pellets were harvested and resuspended with sterile water to achieve an OD_{600} of 0.5. Ten ml of this suspension was then added to the soil around plant roots. *Arabidopsis* were watered by irrigation with 1× Hoagland solution (Hoagland and Arnon, 1950) with and without 200 mM NaCl every third day. *Arabidopsis* pots were filled with vermiculite and perlite containing no nutrients. In the case of tomato, the pots were filled with nutrient-containing horticultural soil and were irrigated with distilled water with or without 200 mM NaCl every three days. After one month, the plant height, fresh weight, and dry weight were measured.

Isolation and taxonomical identification of bacteria

One gram of rhizosphere soil including root tissues of sea china pink (*Dianthus japonicus thunb*), a halophyte plant species, in reclaimed land of Gyehwa-do, Korea were ground in a mortar and inoculated in a flask containing 50 ml of Dworkin-Foster (DF) salt minimal medium (Dworkin and Foster, 1958) mixed with 0.2% ACC (v/v). The flask was incubated on a rotary shaker at 180 rpm for 48 h at 25°C. Subsequently, serial dilutions (10^{-5} to 10^{-6}) were plated again on DF salt minimal agar medium containing 0.2% ACC. After being incubated at 25°C for 2 days, distinct colonies were selected and subjected to further characterization.

For taxonomical identification of the finally chosen bacteria, the total genomic DNA of bacteria was extracted using a DNA extraction kit (GeneAll, Korea). The 16S rRNA gene was amplified by PCR using universal primers, 27F (5′-AGAGTTTG ATCATGGCTCAG-3′) and 1522R (5′-AAGGAGGTGATCCAG CCGAC-3′). The amplified products were then subjected to gel extraction and submitted for nucleotide sequencing.

Measurement of IAA production and ACC deaminase activity

The production of IAA was determined according to the method of Gordon and Weber (1951). Bacteria were inoculated in DF minimal medium supplemented with 0.05% L-tryptophan as an IAA precursor. Samples were cultured at 30°C for 48 h, and then cell cultures were centrifuged at $13{,}000 \times g$. The supernatant was filtrated and then mixed with 150 ml of concentrated H_2SO_4, 250 ml of distilled water, and 7.5 ml of 0.5 M $FeCl_3 \cdot$

$6H_2O$. After 30 min of incubation at room temperature, the absorbance at 535 nm was measured, and the concentration was determined based on a standard curve drawn with a series dilution of IAA (Sigma-Aldrich, USA).

The activity of ACC deaminase was also colorimetrically estimated by measuring the amount of α-ketobutyrate produced from the cleavage of ACC (Penrose and Glick, 2003). Bacterium was cultured in LB broth overnight at 30°C. The cell pellet was retrieved and transferred to DF salt minimal medium containing 3 mM ACC as the sole nitrogen source. When growth reached the late log phase (i.e. $OD_{600} = 1.0$), bacterial cell pellets were harvested, followed by two washes with 0.1 M Tris-HCl, pH 7.6 and pH 8.5, respectively. Toluene was added to the cell suspension and then immediately assayed for ACC-deaminase activities. To create a standard curve, 200 μl of α-ketobutyrate solutions at known concentrations were mixed with 300 μl of 2% 2,4-dinitrophenylhydrazine dissolved in 2 M HCl. The mixture was vortexed and incubated at 30°C for 30 min. Phenylhydrazone color was developed by the addition of 2.0 ml of 2 M NaOH and then measured for absorbance at 540 nm.

Bacterial colonization

Bacterial strains expressing GFP were generated by four-parental mating conjugation. The recipient strain (i.e. *Enterobacter* sp. EJ01), *E. coli* XL1-Blue containing the plasmid pBK-miniTn7-*gfp*2 (Koch et al., 2001), *E. coli* SM10/λ *pir* containing the plasmid pTNS3, and the helper strain *E. coli* HB101 containing the plasmid pRK2013 were grown separately overnight in LB broth in the presence of relevant antibiotics at 37°C with shaking. 100 μl of each cell culture was taken and washed twice with LB pre-warmed to 37°C. The cultures were combined and spotted on pre-warmed solid LB and incubated at 37°C for 6 h. Cells were suspended using saline solution (0.85% NaCl) and plated on LB-agar supplemented with gentamycin (200 μg/ml: selection for pBK-miniTn7-*gfp*2 insertion) and ampicillin (30 μg/ml: for EJ01) and incubated overnight at 37°C (Choi and Schweizer, 2006). Final colonies were selected, and their 16S rRNA genes were amplified and sequenced for confirmation. Selected bacterial strains were grown overnight in LB media, and cell pellets were resuspended to achieve OD_{600} = 0.5 with deionized water. Cell suspensions were administered to *Arabidopsis* seedlings grown vertically in a plate. The entirety of the roots was examined for GFP fluorescence using an AX10 fluorescent microscope (Zeiss, Sweden) or LSM 510 META confocal laser scanning microscope (Zeiss, Sweden).

Quantitative measurement of expression of stress responsive genes

Total RNA was isolated from whole seedlings treated with bacterial suspension (OD_{600} = 0.5) for six h, using TRIzol reagent (Invitrogen, USA). The first strand of cDNA was synthesized using one microgram of total RNA as a template and oligo dT primers (Takara, Japan). Diluted cDNAs were used as templates for real-time qRT-PCR using SYBR® *Premix Ex Taq*™ (Takara, Japan) with a Rotor-Gene 6000 real-time rotary analyzer (Corbett Life Science, Australia). The expression levels of target genes were presented as a ratio relative to the expression of the reference gene *ACTIN2*.

Measurement of ROS scavenging activity

ROS scavenging activities were assessed by measuring DPPH and APX activities. The neutralization of DPPH, a stable radical, was determined by a modification of a previously described protocol (Blois, 1958). A reaction mixture containing 1.5 ml of 0.2 mM DPPH dissolved in 80% ethanol and 300 μl of plant extract was incubated at 25°C for 30 min, and then the absorbance at 540 nm was measured using a Shimadzu spectrophotometer UV-1800 (Shimadzu, Japan). All measurements were performed in triplicate. DPPH radical scavenging activity (expressed as a percentage) was calculated as $[(Abs_{DPPH}-Abs_{sample})/Abs_{DPPH}] \times 100$.

The activities of APX were determined as described (Nakano and Asada, 1981). 0.5 g of the aerial parts of tomato plants were ground into fine powder, and the total proteins were extracted with 750 μl of 50 mM potassium phosphate buffer at pH 7.5 and 10 mM ascorbate in the presence of 20 mg of PVPP (Polyvinylpolypyrrolidone). After vigorously vortexing for 10 min, the mixture was centrifuged at 12,000 rpm for 15 min at 4°C, and the supernatant (protein extract) was taken. The enzymatic reaction was initiated by adding 1372 μl of 50 mM K-phosphate at pH 7.5, 75 μl of 10 mM ascorbate, 3 μl of 100 mM H_2O_2, and 50 μl of protein extract. The oxidation of ascorbate to dehydroascorbate was monitored colorimetrically by measuring the absorbance at 290 nm. The activity was calculated as follows: APX activity (μmol/g fresh weight/min) = (Δ290/min) × [Total enzyme extraction buffer volume / volume of enzyme solution in reaction mixture] × (volume of reaction mixture) / 2.8 (molecular extinction coefficient) × [1/weight of sample tissue (g)].

Statistical analyses

The significance of difference between bacterially infected and uninfected conditions in the growth promotion and salt tolerance of *Arabidopsis* and tomato were analyzed by student's *t*-tests. The quantitative real-time PCR of stress-responsive genes and ROS scavenging activities were assessed by the standard deviation of mean values from triplicates.

RESULTS

Isolation of *Enterobacter* sp. EJ01

To discover bacteria that could enhance the growth and salt resistance of host plants, we isolated bacteria from the rhizosphere of a halophytic plant, sea china pink, and screened for ACC-utilizing capability, a well-documented PGPR trait (Bulgarelli et al., 2013). As a result, isolate 4-1 exhibited the best growth in medium containing ACC (Supplementary Fig. 1), implying high levels of ACCD (ACC deaminase) production. The enzymatic activity of ACCD from isolate 4-1 was measured to be 27.09 μmole α-ketobutyrate/mg of proteins of cell pellet after 16 h of growth in the presence of ACC as the sole nitrogen source. We also examined whether isolate 4-1 could produce the auxin. Noticeably, when grown in broth culture, isolate 4-1 secreted and accumulated IAA in proportion to incubation time (48.3 mg of IAA/ml of broth after 24 h of incubation). On the other hand, siderophore activity, known to facilitate iron uptake in roots, was rarely detected in the growth medium of 4-1 (data not shown).

To taxonomically identify isolate 4-1, its 16S rRNA gene was amplified and sequenced. BLASTN (http://blast.ncbi.nlm.nih.gov) revealed that isolate 4-1 shared 99% homology with *Enterobacter* sp. R4-422 (GenBank accession no.: JQ659709.1) and *Enterobacter* sp. R4-368 (GenBank accession no.: CP005991). Based on this, we designated isolate 4-1 as *Enterobacter* sp. EJ01.

Enterobacter sp. EJ01 promotes growth and increases salt resistance of tomato seedlings

To test whether *Enterobacter* sp. EJ01 exerted PGPR activities

and modulated plant responses against abiotic stresses, we infected tomato seedlings with EJ01 and monitored the growth behavior in the presence or absence of 200 mM NaCl. After growing for one month, tomato seedlings infected with EJ01 displayed increased biomass, as determined by measurement of the length, fresh weight, and dry weight of the aerial parts of the plants (Fig. 1A). Compared to uninfected seedlings, EJ01-infected tomatoes exhibited 37% and 20% increases in plant height under normal and high salinity conditions, respectively (Fig. 1B). More dramatic differences were observed in the biomass accumulation. Under normal conditions, EJ01 infection conferred increases in the fresh weight (49%) and dry weight (50%) of uninfected tomatoes (Figs. 1C and 1D). In salt-stressed conditions driven by 200 mM NaCl, EJ01-infected seedlings still showed over 20% increases in both fresh and dry weight measurement compared to uninfected seedlings. The combined data strongly suggest that EJ01 promoted growth and alleviated the damage driven by salt stress in tomato seedlings.

Enterobacter sp. EJ01 confers facilitated growth and increased salt resistance to *Arabidopsis* seedlings

To further confirm the effect of EJ01 on the growth and salt stress tolerance of host plants, we extended our physiological studies to *Arabidopsis*. After growing for one month, *Arabidopsis* seedlings infected with EJ01 displayed increased biomass, as assessed by fresh weight and dry weight (Fig. 2A). Under stress-free conditions, the fresh weights of *Arabidopsis* Col-0 seedlings in EJ01-uninfected and -infected conditions were 0.28 g and 0.38 g, respectively, a 30% difference (Fig. 2B). This clearly suggested that EJ01 infection boosted the vegetative growth of *Arabidopsis*. In the presence of 200 mM NaCl, EJ01-infected Col-0 seedlings also revealed higher fresh weight (0.14 g) than uninfected seedlings (0.07 g), representing a 100% increase. Dry weight measurement also showed similar results to the fresh weight. Compared to uninfected conditions, EJ01-infected Col-0 seedlings exhibited 42% and 72% increases in dry mass under non-stressed conditions and with 200 mM NaCl, respectively (Fig. 2C). The combined results strongly suggest that EJ01 promotes growth and alleviates salt stress in *Arabidopsis*.

Enterobacter sp. EJ01 up-regulates the expression of stress-responsive genes in the early stages of infection

To delineate the mutualistic interaction between PGPRs and their host plants at the molecular level, the expressions of marker genes up-regulated in ABA-dependent and/or -independent pathways in *Arabidopsis* were examined after a short period of bacterial infection (Fig. 3). Six hours after exposure to salt, the expressions of *DREB2b, RD29A, RD29B,* and *RAB18* were increased in the EJ01-infected *Arabidopsis* seedlings compared to the uninfected. Interestingly, *DREB2b*, *RD29A*, and *RAB18* in EJ01-infected seedlings were up-regulated, even in the absence of salt treatment. On the other hand, increases in *RD29B* transcript levels in EJ01-infected seedlings were dependent on salt treatment. We also examined the expression levels of *MPK3* and *MPK6,* which were reported to regulate the 'priming' processes in biotic stress resistance (Beckers et al., 2009). Interestingly, EJ01 infection alone increased the expressions of *MPK3* and *MPK6*. This up-regulation of *MPK3* and *MPK6* was marginal but statistically significant. However, after salt treatment, no difference was observed between EJ01-free and EJ01-infected conditions. Taken together, EJ01 may positively activate MAPK signaling at the transcriptional level but in a limited scale.

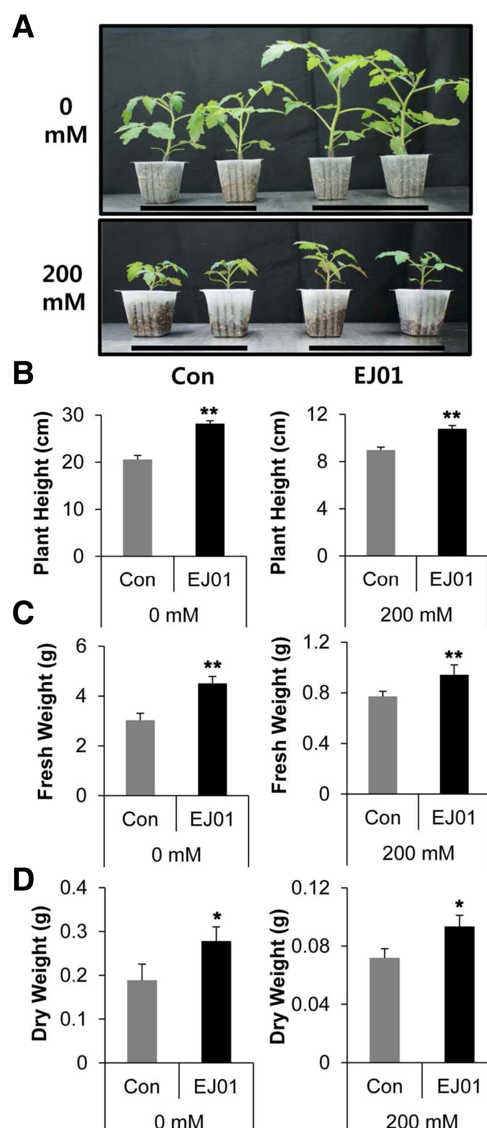

Fig. 1. The effects of *Enterobacter* sp. EJ01 on growth and salt resistance in tomato. (A) Representative tomato seedlings, uninfected or infected with EJ01, grown in the presence or absence of 200 mM NaCl. (B) Average lengths of aerial parts of tomatoes under the same conditions. (C) Average fresh weight of tomato seedlings under the same conditions. (D) Average dry weight of tomato seedlings under the same conditions. Error bars indicate standard deviation (n = 16). *$P < 0.05$; **$P < 0.01$ for Student's *t*-tests. After germination, tomato seedlings were transplanted to pots and treated with EJ01 suspensions near the root zone. Plants were irrigated with distilled water with or without 200 mM NaCl every three days. After one month, tomato seedlings were photographed, and then their aerial parts were removed and measured for fresh weight. Dry weight of aerial parts was measured after 2 days in a drying oven at 75°C. Con: No bacterial infection; EJ01: EJ01-infected seedlings.

The accumulation of compatible solutes such as sugars, glycine betaine, and proline is also a common stress avoidance mechanism in *Arabidopsis*. To investigate whether proline syn-

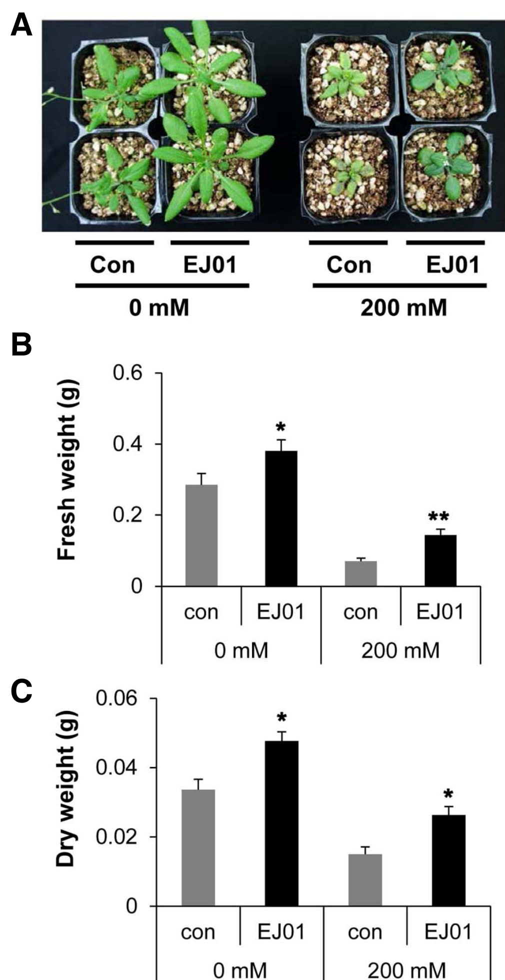

Fig. 2. The effect of *Enterobacter* sp. EJ01 on growth and salt tolerance of *Arabidopsis.* (A) Representative seedlings grown in pots treated or untreated with EJ01, under 0 mM and 200 mM NaCl. (B) Average fresh weight of individual *Arabidopsis* seedlings grown under the same conditions. (C) Average dry weight of individual *Arabidopsis* seedlings grown under the same conditions. Error bars indicate standard deviation (n = 25). *P < 0.05; **P < 0.01 for Student's *t*-tests. *Arabidopsis* seedlings germinated on MS plates were transferred into pots and then treated with an EJ01 bacterial cell suspension or sterilized water. After two days, pots were watered by irrigation with 0.5× Hoagland nutrient solutions containing either 0 mM or 200 mM NaCl every three days. Bacterial infection was repeated once more on the seventh day. After one month in pots, the *Arabidopsis* specimens were photographed. The aerial parts were then taken for measurement of the fresh weight. For dry weight determination, the seedlings taken for fresh weight measurement were dried at 70°C for two days and then weighed. Con, No bacterial treatment; EJ01, EJ01-treated seedlings.

thesis is facilitated by the EJ01 strain, we assessed the expression levels of *P5CS1* and *P5CS2* genes, which encode rate-limiting steps in proline biosynthesis. Notably, the expression of *P5CS1* was highly up-regulated in EJ01-infected plants under stressed conditions. Interestingly, however, *P5CS2* was also up-regulated by salt treatment but showed no difference between bacteria-free and EJ01-infected conditions. On the other hand, the expression of *PR1*, the most reliable read-out of the activation of defense, was also up-regulated, suggesting that EJ01 activated the innate immunity pathways as well as salt-stress-responsive signaling.

ROS scavenging activities are increased in *Arabidopsis* seedlings exposed to *Enterobacter* sp. EJ01

Prolonged salt stress can cause oxidative stress and produce ROS, including superoxide, singlet oxygen, hydroxyl, and hydrogen peroxide inside cells. Such stressors can be countered by various antioxidants and enzymes directly related to stress resistance that detoxify ROS. To further explore the beneficial effects of EJ01 on the stress tolerance of host plants at a mechanistic level, the activities of common ROS scavenging systems in plants were quantitatively measured (Fig. 4). We first measured the activity of APX (ascorbate peroxidase), which is known to play key roles in detoxification under combinatorial stress (Koussevitzky et al., 2008) (Fig. 4A). EJ01-treated seedlings exhibited higher APX activities (3.2 μmol/min/g fresh weight) under stressed conditions than did EJ01-free seedlings (2.6 μmol/min/g fresh weight). Similarly, DPPH (1,1-diphenyl-2-picrylhydrazyl), a widely used radical for antioxidant assays, was also neutralized more in EJ01-treated seedlings (55%) than in EJ01-free seedlings (46%) (Fig. 4B). Taken together, EJ01-treated plants activated their detoxification processes against oxidative conditions driven by salt stress more efficiently than did untreated plants.

Spatial distribution of *Enterobacter* sp. EJ01 in host plant

Next, to determine whether EJ01 colonized the interior of host plants, we monitored Green Fluorescent Protein (GFP) signals in the root tissues of *Arabidopsis* infected with recombinant EJ01 strains expressing *GFP*, which were generated by four-parental mating conjugation (see "Materials and Methods"). When *Arabidopsis* was treated with wild-type EJ01, no fluorescent signal was detected throughout the entire root system (data not shown). In contrast, *Arabidopsis* infected with GFP-expressing EJ01 strains (Fig. 5) exhibited strong GFP signals in the various regions in the roots. After one day of infection, EJ01 strains were colonized dominantly in the rhizosphere (Fig. 5A). However, after two days of infection, EJ01 were observed in the apoplastic regions of the maturation zone (Figs. 5B and 5C), the vasculatures (Figs. 5D and 5E), or the cytosolic compartment (Figs. 5F and 5G) in the roots. In some cases, EJ01 was specifically crowded around cells and appeared to intrude into the host tissue (Supplementary Fig. 2). On the other hand, no GFP signal was detected in the shoots (data not shown). Overall, EJ01 was distributed in the host tissue with indefinite spatial preference, indicating that EJ01 was dominantly rhizobacteria but also exhibited endospheric properties.

DISCUSSION

Many bacteria and fungi in the rhizosphere, known as PGPRs, facilitate the growth of their host plants (Kloepper and Schroth, 1978). Interestingly, when host plants are pre-treated (i.e. 'primed') with some bacteria, the host basal innate immunity can be increased, along with the induction of rapid protective responses to genuine pathogenic attacks at a systemic level, a phenomenon referred to as induced systemic resistance (ISR) (Kloepper et al., 2004). In addition, some PGPRs also confer resistance to abiotic stresses like salinity (Yang et al., 2009). Most previous studies have explicitly focused on the physiologi-

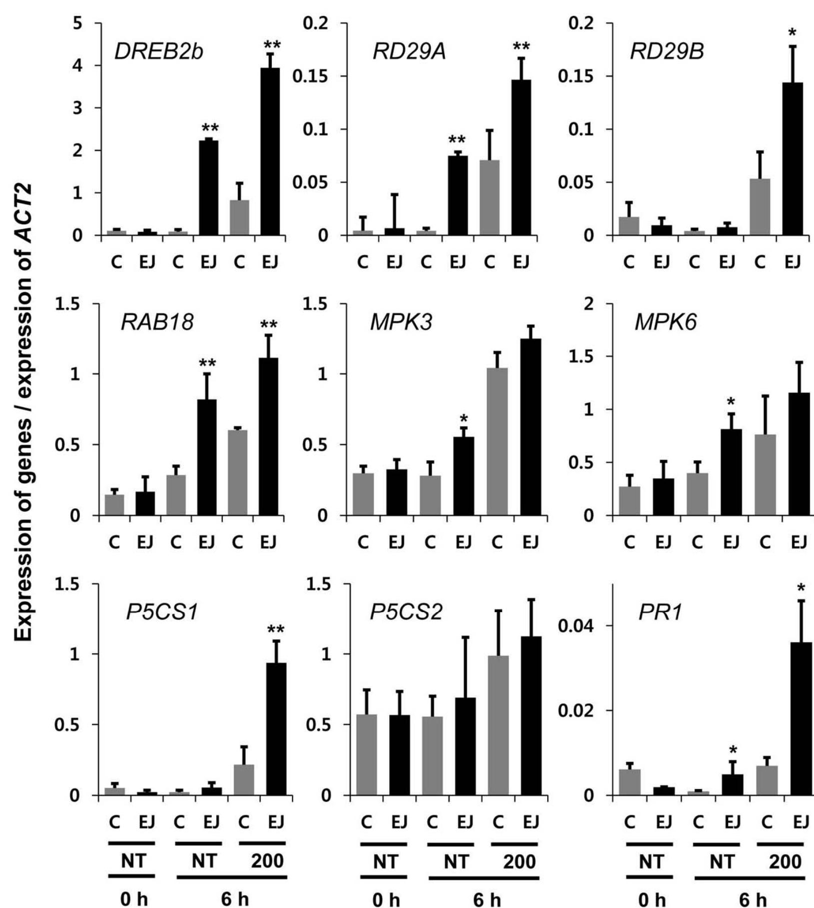

Fig. 3. Quantitative measurements of the expression levels of marker genes regulating stress responses in *Arabidopsis.* Ten-day-old *Arabidopsis* seedlings were treated with *Enterobacter* sp. EJ01 under 0 mM or 200 mM NaCl (see "Materials and Methods"). After six h, whole seedlings were collected and subjected to RNA isolation, followed by quantitative real-time RT-PCR. *ACT2* was used as a control. Error bars indicate standard deviation (n = 3). *$P < 0.05$; **$P < 0.01$ for Student's *t*-tests. C, No bacterial treatment; EJ, EJ01-treated seedlings; NT, No addition of NaCl; 200, addition of 200 mM NaCl. 0 h, Zero time point of bacterial infection; 6 h, six hours after bacterial infection.

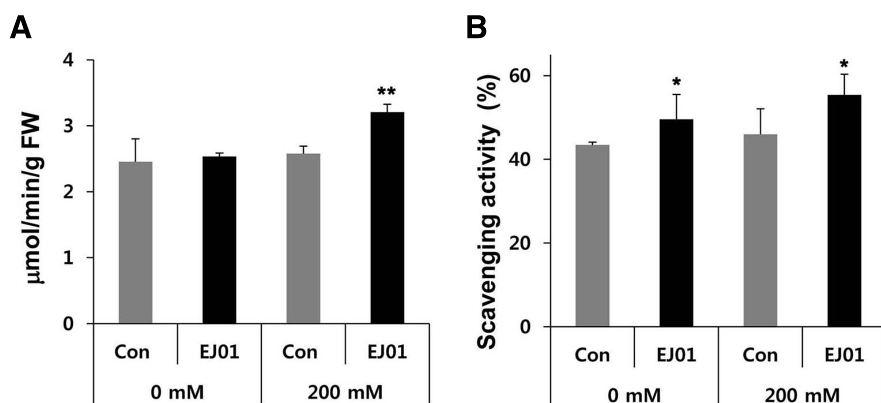

Fig. 4. The effect of strain EJ01 on ROS scavenging activities of tomatoes. (A) Activity of APX present in tomato seedlings. (B) Reduction of DPPH by tomato seedling extracts. Protein extracts from the aerial parts of one-month-old tomatoes were used in APX and DPPH activity assays. For APX activity measurement, the reduction of hydrogen peroxide was monitored by the oxidation of ascorbate to dehydroascorbate. For DPPH, the reduction of DPPH was monitored colorimetrically, and scavenging activities were calculated as percentage decrease in DPPH. Error bars indicate standard deviation (n = 3). *$P < 0.05$; **$P < 0.01$ for Student's *t*-tests. Con, No bacterial infection; EJ01, EJ01-infected tomato.

cal aspects of these effects, but the molecular basis thereof has not been investigated. Our study demonstrated that the newly isolated *Enterobacter* sp. EJ01 was capable of enhancing the growth and salt resistance of tomatoes and *Arabidopsis*. More importantly, stress tolerance driven by EJ01 is likely accompanied by bacteria-primed transcriptional activation of multiple stress-responsive factors prior to exposure to salt stresses, in a fashion similar to ISR against attacks by pathogens.

The effects of EJ01 on phytohormone production

EJ01 exhibits common PGPR characteristics such as ACCD and IAA production, suggesting that the growth promotion driven by EJ01 is likely ascribed to an increased level of auxin and the reduction of ethylene in the host plant. Combined genetic

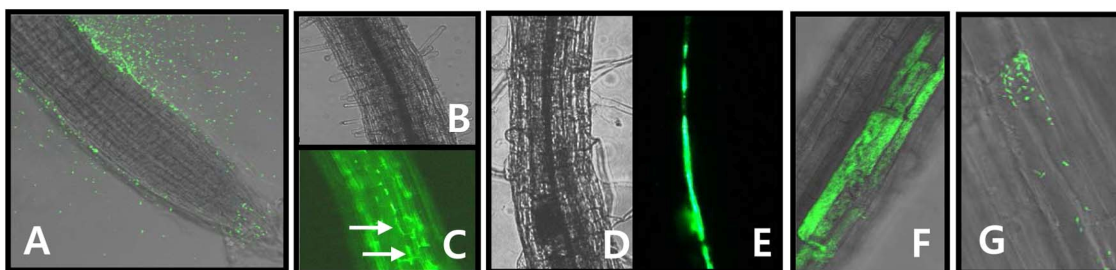

Fig. 5. Root colonization of recombinant *Enterobacter* sp. EJ01 expressing *GFP*. (A) The bacterial distribution in a longitudinal layer of rhizosphere zone. After one day of infection, meristemic zones of *Arabidopsis* root were monitored by optical sectioning. (B, C) The maturation zone of the roots. The arrows indicate the apoplastic regions or other cell periphery. (D, E) The vasculature of the maturation zone of the roots. (F) The cytosolic compartment of cell in elongation zone. (G) The enlarged image of one cell colonized by EJ01. (A, F, and G), observed by confocal fluorescent microscope; (B-E), observed by standard fluorescence microscope. GFP-containing EJ01 strains were generated by four parental mating (see "Materials and Methods") and applied to two-week-old *Arabidopsis* seedlings vertically grown in MS plate. After one and two days, GFP signals in the roots were monitored under fluorescent microscope or confocal laser scanning microscope. To prepare mating conjugation, the recipient strain EJ01, XL1-Blue *E. coli* containing the plasmid pBK-miniTn7-*gfp*2 (Koch et al., 2001), *E. coli* SM10/λ *pir* containing the plasmid pTNS3, and the helper strain *E. coli* HB101 containing the plasmid pRK2013 were used.

studies have demonstrated that the production of IAA or auxins from bacteria functionally correlates with increased growth of the host plants (Spaepen et al., 2007). For *A. brasilense*, a direct role for IAA production in the root architecture was demonstrated in wheat. In our study, EJ01 produced 43 mg/ml of IAA in liquid broth media, which was comparable with the amount produced by *Klebsiella oxytoca* Rs-5 (42.14 mg/ml broth), a bacterial strain that increases salt stress tolerance in cotton (Liu et al., 2013). This could be interpreted that auxin produced by EJ01 might be responsible for growth promotion and salt tolerance. However, this must be regarded as uncertain, because auxin biosynthesis by several bacteria (e.g. *Piriformospora indica*) has been shown to be necessary only for root colonization (Hirano and Upper, 2000). In addition, *Bacillus subtilis* GB03 is known to facilitate the growth of host plants by inducing synthesis of auxin by its host, and not *via* IAA produced by itself (Zhang et al., 2007). Therefore, it is still unclear whether IAA produced by EJ01 is directly responsible for the observed growth promotion.

ACCD production has also been proposed as a key beneficial factor in PGPR action (Glick et al., 1998) and used as a selection criterion in primary screening for many PGPRs. In the current study, EJ01 was also isolated through screening for ACC utilization, and its ability to produce ACCD was confirmed. Previous reports demonstrated a correlation between ACCD production and the alleviation of salt stresses in host plants, but a direct correlation was not investigated on a wide scale (Mayak et al., 2004a; 2004b). *Bacillus pumilus* WP8 and *Pseudomonas putida* RBP1 fail to secrete ACCD, although they do increase salt tolerance (Shen et al., 2012). These findings imply that reduced ethylene production is not the sole factor accounting for salt tolerance in the strain EJ01.

The roles of EJ01 in host stress adaptive signaling

Under freezing and salt stresses, the activation of MKK2 (mitogen-activated kinase kinase 2) up-regulates the expression of stress-inducible marker genes and increases stress tolerance *via* MPK6 (Teige et al., 2004). The accumulation of mRNA of *MPK3* and *MPK6* was reported to be associated with ISR, which makes plants respond to (a)biotic stresses more rapidly and strongly (Beckers et al., 2009; Teige et al., 2004). Such actions are known to be primed by pre-exposure to moderate (a)biotic stimuli or stresses. This has been supported genetically by the impaired activation of *MPK6* in *mkk2* null plants and severe stressed phenotypes (Teige et al., 2004). In our study, short-term treatment of *Arabidopsis* seedlings with EJ01 increased the expression levels of *MPK3* and *MPK6,* even in the absence of NaCl (Fig. 3), which supported the hypothesis that pre-exposure to bacteria might induce pre-activation of stress-responsive pathways. Such molecular changes would expedite adaptive processes once genuine abiotic stresses are engaged. However, considering the marginal increase in expression levels, the impact of such activation is largely speculative. Rather, the monitoring the status of post-translation modification would provide the better insights into physiological relevance of priming processes activated by EJ01.

The responses of plants to high salinity stresses are exemplified in the model plant, *Arabidopsis* (Zhu, 2002). The PGPR strain *Paenibacillus polymyxa* has been demonstrated to enhance the drought tolerance of *Arabidopsis* by stimulating the transcription of a drought-response gene, *ERD15*, and an ABA-responsive gene, *RAB18* (Timmusk and Wagner, 1999). These genes are known as late embryogenesis abundant (*LEA*) genes, whose expression is commonly up-regulated by cellular water deficits. *LEA* genes have been proposed to be involved in the protection and repair of biomolecules inside the cell. Interestingly, *RAB18* was up-regulated even under PGPR treatments in the absence of salt stresses (Timmusk and Wagner, 1999). Currently, even though no precise mechanism is available, bacterial treatments to the roots could be perceived as a local dehydration-like signal which triggers salt-responsive pathways. Similar to these, in our study, EJ01 increased the expression of *LEA* genes (e.g. *RAB18* and *RD29A*) and their transactivator (i.e. *DREB2b*), even in the absence of NaCl (Fig. 3). These results implicated that moderate exposure to biotic agents might activate signaling components located in the converged points governing multiple (a)biotic stress adaptation processes. On the other hand, the expression of *RD29B*, another *LEA* gene, was increased only when salt stresses were engaged. Differential expression patterns among *LEA* genes might be related to ABA-dependency. Previously, the expression of *RD29A* was known to be highly responsive to drought and cold stress-

ses in both an ABA-dependent and ABA-independent fashion, whereas the promoter of *RD29B* was regulated only by an ABA-dependent pathway (Msanne et al., 2011). In addition, *DREB2b*, a transcription factor of both RD29 genes, displayed ABA-independent activation under osmotic stress conditions (Lata and Prasad, 2011; Zhu, 2002). Based on this, less induction of *RD29B* in EJ01-treated conditions (Fig. 3) implied that EJ01 activated salt-responsive pathways in ABA-independent fashion. Noticeably, this speculation was further supported by no increase in the expression levels of *AAO3*, an ABA biosynthetic gene, when EJ01 was treated (Supplementary Fig. 3). In any event, this speculation must be experimentally examined, but the current results at least indicate that EJ01 activates early signaling events in stress-responsive pathways, providing benefits to host plants under salinity stress. Moreover, the expression of *PR1* is also increased in EJ01- and NaCl-treated seedlings (Fig. 3), suggesting that EJ01 treatment may also enhance innate immunity. Subsequently, EJ01 probably interferes with the upstream components of signaling-responsive pathways, which might impinge upon multiple pathways in (a)biotic stresses.

Turning to physiological aspects, some PGPRs produce antioxidants such as catalase and counter ROS-driven oxidative stresses. *Bacillus safensis* and *Ochrobactrum pseudogregnonense* can withstand osmotic stress *via* the elevation of antioxidant responses (Chakraborty et al., 2013). In our study, EJ01-treated seedlings displayed slightly elevated ROS scavenging activities (Fig. 4), suggesting that the enhanced stress resistance conferred by EJ01 is at least partially due to cellular detoxification processes acting to counter oxidative stresses driven by high salinity. Proline accumulation is also a typical stress response induced by salt, and it is believed to function as an osmolyte or ROS scavenger. Even though proline biosynthesis was assayed only at the level of transcription, the expressions of *P5CS1* and *P5CS2* were greatly boosted in EJ01-exposed seedlings (Fig. 3). Proline accumulation levels have been shown to correlate highly with the transcript levels of *P5CS* genes, which regulate the rate-limiting step in proline biosynthesis (Yoshiba et al., 1997). However, the expression patterns of *P5CS1* and *P5CS2* were quite different from each other. Szekely et al. (2008) reported that the transcription of the *P5CS1* was involved in abiotic stress adaptation. However, *P5CS2* were more likely related to developmental processes such as embryo maturation. At any event, the up-regulation of *P5CS* genes indicates that EJ01 infection increases proline accumulation in *Arabidopsis* seedlings under high-saline cells.

Interestingly, our study clearly shows that EJ01 colonized the roots, and its modes of action must be endophytic (Fig. 5). However, considering the limited existence of EJ01 in the narrow zone of the rhizosphere and/or endosphere, it is intriguing how EJ01 exerted beneficial effects at the whole plant level due to the lack of a definite colonization pattern. Based on our results, EJ01 probably intrudes into plant tissue without spatial preference. In addition, the absence in the shoot tissues implicates that EJ01 might excrete elicitors to activate host signaling, which could be transmitted long distances to have a systemic impact at the whole plant level.

In conclusion, *Enterobacter* sp. EJ01 exerts beneficial roles in plant growth under unstressed and salt-stressed conditions. With respect to its molecular aspects, the mutually interactive modes of action in EJ01-facilitated growth and stress resistance in *Arabidopsis* and tomato include early responses activating osmolyte accumulation, ROS scavenging, and protein repair and protection driven by *LEA* genes. Monitoring the beneficial effects of EJ01 using appropriate mutant allelic plants of which stress signaling were modified would further elaborate its underlying mechanisms.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

ACKNOWLEDGMENTS

This research was supported by the National Research Foundation of Korea (NRF) grant funded by the Korean government [Ministry of Education, Science and Technology (MEST)] (No. 2011-0020202) and the research funds of Chonbuk National University in 2013. The authors especially thank Gun-woong Lee, Kyeong-Taek Kim, and Eun-Ji Kim for technical assistance.

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