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Aluminium resistant, plant growth promoting bacteria induce overexpression of Aluminium stress related genes in *Arabidopsis thaliana* and increase the ginseng tolerance against Aluminium stress

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ABSTRACT

Panax ginseng is an important cash crop in the Asian countries due to its pharmaceutical effects, however the plant is exposed to various abiotic stresses, lead to reduction of its quality. One of them is the Aluminum (Al) accumulation. Plant growth promoting bacteria which able to tolerate heavy metals has been considered as a new trend for supporting the growth of many crops in heavy metal occupied areas. In this study, twelve bacteria strains were isolated from rhizosphere of diseased Korean ginseng roots located in Gochang province, Republic of Korea and tested for their ability to grow in Al-embedded broth media. Out of them, four strains (*Pseudomonas simiae* N3, *Pseudomonas fragi* N8, *Chryseobacterium polytrichastri* N10, and *Burkholderia ginsengiterrae* N11-2) were able to grow. The strains could also show other plant growth promoting activities *e.g.* auxins and siderophores production and phosphate solubilization. *P. simiae* N3, *C. polytrichastri* N10, and *B. ginsengiterrae* N11-2 strains were able to support the growth of *Arabidopsis thaliana* stressed by Al while *P. fragi* N8 could not. Plants inoculated with *P. simiae* N3, *C. polytrichastri* N10, and *B. ginsengiterrae* N11-2 showed higher expression level of Al-stress related genes, *AtAIP*, *AtALS3* and *AtALMT1*, compared to non-bacterized plants. Expression profiles of the genes reveal the induction of external mechanism of Al resistance by *P. simiae* N3 and *B. ginsengiterrae* N11-2 and internal mechanism by *C. polytrichastri* N10. Korean ginseng seedlings treated with these strains showed higher biomass, particularly the foliar part, higher chlorophyll content than non-bacterized Al-stressed seedlings. According to the present results, these strains can be used in the future for the cultivation of ginseng in Al-persisted locations.

1. Introduction

Many of Agricultural soil area are contaminated with many of heavy metals (Ghnaya et al., 2010) and Aluminum (Al) is considered one of them (Goodwin and Sutter, 2009). Although Al is required for plant growth, it has toxic effects on the plant development in the acidic soil (Ezaki et al., 2004) which represent around 30% of the total earth's lands and as much as 50% of the total arable lands. Most of acidic soil in the tropical and subtropical regions are extensively used for agricultural purpose, therefore Al toxicity in these regions represent a serious threat (Von Uexküll and Mutert, 1995). The toxicity of Al affects the crop productivity by limiting the root elongation, which accordingly reduces the uptake of the nutrient and water from the soil, leading to the reduction of the growth of the whole plant (Kochian, 1995; Goodwin and Sutter, 2009; Ma et al., 2012). Many physiological mechanisms have been reported by which the plant tolerates the toxicity of Al. They are divided into external and internal tolerance. External tolerance is accomplished by the production of organic acids from the roots cell to the rhizosphere in order to chelate surround Al ions and make it unavailable to the plant (Magalhaes et al., 2007; Ryan et al., 2011; Delhaize et al., 2012). The internal tolerance occurs by the dragging of Al ions and trapped in plant cell walls or plasma membrane or inside the plant cell in the vacuoles away from sensitive tissues (Kochian, 1995; Ramgareeb et al., 2004). Al-tolerant related genes have been characterized as summarized in Fig. 1; *ALMT1* (Aluminum-activated maleate transporter 1) is a gene which encodes malate transporter which participated in the tolerance of the plant against Al stress by transporting the organic acid, malate out of the plant cell to chelate rhizospheric Al^{3+} and make it unavailable to the plant uptaking (Ryan et al., 1995; Raman et al., 2005; Ryan et al., 2011; Delhaize et al., 2012;

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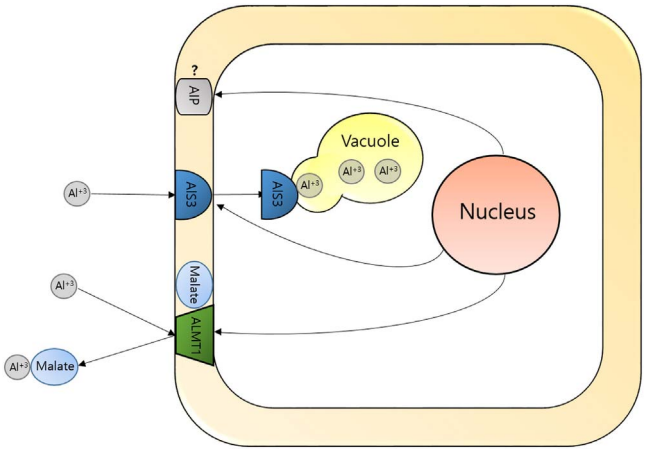

Fig. 1. A schematic demonestrating the propable tolerance mechanisms shown by the plant cell under the condition of Al stress. Abbreviations; Al, Aluminium.

Ma et al., 2012). *ALS3* (Aluminum sensitive 3) is a gene which encodes ABC transporter-like protein located in the plasma membrane of the phloem and works in redistributing the accumulated Al inside plant away from the sensitive tissues (Larsen et al., 2005). *AIP* (Aluminum induced protein) is a protein associated with plant tolerance against Al stress, however the function of it in the tolerance mechanism still unclear (Richards et al., 1994; Jang et al., 2014). Recently, many transgenic plants holding these genes have been produced in order to increase the tolerance of the plant against Al toxicity in the acidic soils (De la Fuente-Martínez and Herrera-Estrella, 1999; Pereira et al., 2010).

Panax ginseng is a slow growing perennial plant belongs to Araliaceae family. It has been cultivated for many years ago due to its valued roots which has been used as a curative drug and as a health tonic in traditional Asian medicine (Kim et al., 2014). Recently, ginseng extracts have been intervened in many health care products *e.g.* capsules, drinks and cosmetics. These pharmaceutical activity has been found to belong the main secondary metabolites exist in the roots called ginsenosides (Siraj et al., 2015). Good quality of ginseng root needs 4–6 years cultivation under special caring and observation (Yang, 1992) in weakly acidic or neutral soils while bad yield has been obtained from acidic soils (Lee, 2007; Hankins, 2009). Rusty roots were observed to have reduced levels of ginsenosides. Furthermore, elevated amount of Fe and Al were detected in the rusted tissues compared to the adjacent healthy tissues (Rahman and Punja, 2005). Later, foliar application of isotopic Fe revealed the role of the iron in the increase of plant susceptibility to the pathogens infection (Rahman and Punja, 2006). The role of Al has not yet confirmed, but the chemical analysis of diseased ginseng soil showed higher accumulation of Al element compared to healthy soil (Kernaghan et al., 2007). Furthermore, in the present study, the irrigation of Korean ginseng by Al lead to the reduction of fine root growth and wilting and mass reduction of foliage.

In general, microbial population has a potential factor affecting crops yield and some of them are chosen as fertilizers or pesticides for their reproductive abilities to promote the plant growth and reduce the pathogens invasion. Such selective microbial isolates are called PGPB (Plant growth promoting bacteria). PGPB are bacteria which has special symbiotic relationship with plants (*e.g.*, *Rhizobia* spp. with nodulating plants and *Frankia* spp. with actinorhizal plants), those which are free-living and those live endophytically (Bashan et al., 2004). PGPB are bacterial isolates isolated from different environmental locations and capable of positively affecting plant growth parameters and yields directly or indirectly. Direct effects are accomplished by facilitating the nutrient acquirement from the rhizosphere (*e.g.* solubilizing the insoluble phosphate) or by modulating the plant hormone levels (Kampert et al., 1974; Tsavkelova et al., 2005; Vassilev et al., 2006; Ahmed and Hasnain, 2010). Indirect effects take place by reducing the invasion of plant pathogens by either antibiosis or producing metal-chelating substances called siderophores. Siderophore-producing PGPB also help plants to uptake various metals easily *e.g.* iron, zinc and copper. Furthermore, they reduce the bioavailability of heavy metals which are toxic to plants surround the plant rhizosphere (Dimkpa et al., 2009). Siderophore-producing PGPB can successfully support the growth of the plant in soils contaminated with various types of toxic metals (Belimov et al., 2005; Barzanti et al., 2007; Jiang et al., 2008; Kuffner et al., 2010). Furthermore, PGPB can induce the plant tolerance against the metal stress by the induction of metal stress related genes expression (Sukweenadhi et al., 2015). PGPB which have been reported as metal-tolerant isolates belong to the genera *Variovorax*, *Methylobacterium*, *Burkholderia*, *Okibacterium*, *Rhodococcus*, *Microbacterium*, *Sphingomonas*, *Curtobacterium*, *Serratia*, *Pseudomonas*, *Ralstonia*, *Staphylococcus*, *Bacillus*, *Arthrobacter*, *Paenibacillus*, *Chryseobacterium*, and *Leifsonia* (Idris et al., 2004; Belimov et al., 2005; Dell'Amico et al., 2005; Barzanti et al., 2007; Jiang et al., 2008; Kuffner et al., 2010; Benmalek et al., 2014). Some of them were proposed to be used as supported inoculants to the plants in metal contaminated soils. These bacteria were isolated from either the rhizospheres or the plant tissues of *Thlaspi goesingense*, Graminaceae grasses, *Allysum bertolonii*, *zea mays*, *Salix caprea* and can increase the plant tolerance against nickel, cadmium, lead, and zinc (Belimov et al., 2005; Barzanti et al., 2007; Jiang et al., 2008; Kuffner et al., 2010). According to our knowledge, little is known about Al-resistant PGPB and their role to support plants, particularly ginseng tolerance against Al toxicity. Therefore, the present study aims to isolate Al-tolerant PGPB strains from rhizosphere of Korean ginseng roots which able to support ginseng growth under Al stress.

2. Material and methods

2.1. Molecular identification of the bacteria

The genomic DNA of the bacteria strains was extracted and purified using commercial DNA isolation kit (Gene All, South Korea). 16S rRNA gene sequence was amplified using the primer set including 27F/1492R (Lane, 1991) and 518F/800R (Weisburg et al., 1991). The purified PCR products were sequenced by Genotech Company (Daejeon, Republic of Korea). The resultant sequences were corrected and complied using Seq-Man software version 4.1 (DNASTAR.) and then compared with 16S rRNA gene sequences available in public databases using BLASTN searches on NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgihttp://blast.ncbi.nlm.nih.gov/Blast.cgi) and in the EzTaxon-e server (Kim et al., 2012).

2.2. In vitro estimation of plant growth promoting traits

Production of the plant growth regulating hormone, Indole-3-acetic acid (IAA) was investigated as described by Glickmann and Dessaux (1995) with some modification; each strain was cultured in modified King B broth with and without $_{L}$-tryptophan (3 g/L). IAA production was quantitatively checked after 3 and 6 days incubation time using Salkowski reagent. Siderophore production was qualitatively determined using *Pseudomonas* Agar F (Difco) supplied with a chrome azurol S complex [CAS/iron(III)/hexadeciltrimethyl ammonium bromide] (Schwyn and Neilands, 1987). Qualitative testing of phosphate solubilization was made using Pikovskaya agar (Pikovskaya, 1948; Sigma-Aldrich).

2.3. Determination of Al-resistant bacteria

The ability of the isolated bacteria to resist Al was evaluated as follows. Each strain was cultured in nutrient broth (NB; MB cell) supplied with filter sterilized $Al_2(SO_4)_3$ in different concentrations (2 mM, 4 mM, 8 mM, and 16 mM). The strains were cultured in the

same media but without the addition of $Al_2(SO_4)_3$, as control. After one week, the optical density (OD) of the strains was checked and growth rate was estimated using the following equation [growth rate = $(OD_{control}\text{-}OD_{Al\ treated})/OD_{control}$].

2.4. Arabidopsis thaliana treatment with bacteria

Seeds of *A. thaliana* ecotype Columbia (Col-0) were gifted by Dr. Yu-Jin Kim (Kyung Hee University, South Korea) and used in this experiment. The seeds were sterilized in 20% Clorox for 2 min, followed by 70% ethanol for 1 min. Then, the seeds were washed 5 times with sterilized water and directly sown in 10-cm pots containing sterilized soil. The pots were kept at 4 °C in dark for breaking the seeds dormancy and then transferred to plant growth chamber at 22 °C with a 16-h light photoperiod for 2 weeks until germination. Then, the seedlings were separately transplanted and grown under the same condition for further 2 weeks to be ready for bacterial inoculation. Candidate bacteria for *A. thaliana* inoculation were cultured in *Pseudomonas* Agar F (Difco) plates for 24 h and then dissolved in saline water. The bacteria suspension density was adjusted for each strain until reaching 10^8 CFU/mL and then inoculated to 4 weeks old *A. thaliana* plants; plants representing mock and negative control were treated with saline water only.

2.5. Stress application on A. thaliana

After 5 days of the bacterial treatment, Al stress was conducted by watering of *A. thaliana* plants with 500 mM $Al_2(SO_4)_3$ solution. Twelve pot replication were conducted for each treatment. Growth parameters *e.g.* rate of wilted plants and chlorophyll content were determined after 7 days, where the symptoms of yellowing and wilting appeared on the plant rosettes. Before that time (at the third day), plant samples were collected from each treatment, directly frozen using liquid nitrogen, and kept at −20 °C for RNA extraction and quantification.

2.6. Quantitative real time PCR analysis

Total RNA were isolated from completely frozen *A. thaliana* plants using the RNeasy kit (Qiagen, Germany) according to the manufacturer's instructions. Two μg of isolated RNA were used for synthesis of cDNA using cDNA synthesis kit (Invitrogen, USA) following the instruction given by the manufacturer to the final volume of 20 μL. One μL of synthesized cDNA of each treatment were used for quantitative real time PCR (qRT-PCR) analysis in a reaction volume of 10 μL containing iQ™ SYBR® Green Supermix (Bio-Rad, USA) and Al resistance related gene specific primers. The housekeeping gene, Actin was used as control (Table 3). The thermal cycler condition recommended by the manufacturer were used: 3 min at 95 °C, followed by 39 cycles at 95 °C for 10 s, 55 °C for 30 s, then ended with 95 °C for 10 s and 65–95 °C for 5 s with 0.5 °C increments for melt curve analysis. Fluorescence was detected at the final step at each cycle. Amplification, detection and data analysis were carried out on CFX Manager™ Software version 3.1 (Bio-Rad, USA). To show the relative abundance of the determinant templates, the C_T value of a particular template was normalized to C_T value of the Actin and calculated relative to a calibrator using the formula $2^{-\Delta\Delta C_T}$.

2.7. Stress application on P. ginseng seedlings

Two-year old Korean ginseng seedlings, ready for sprouting were purchased from a private field during February 2016, directly brought to the lab and kept at 4 °C for a short time until the time of the experiment. Then, roots with equal length and width and intact coated rhizome were selected for the stress application. Roots were inserted in 20 cm pots containing sterilized artificial soil mixture and incubated in greenhouse (22 ± 2 °C, 12-h photoperiod) for 3 weeks until full sprouting. Fully sprouted seedlings (5 roots/pot) were selected for the stress application. Strains that successfully induced the phenotypic and molecular tolerance of *A. thaliana* to Al stress were selected for *P. ginseng* treatment; each strain were prepared and applied to ginseng as explained above for *A. thaliana*. After 10 days of the bacterial inoculation, Al stress were applied as explained above. The morphological change were recorded for the foliar part each 2 days and harvesting were carried out after 7 days of Al stress application. Harvested seedlings were divided into roots and foliar parts and used for determination of growth parameters *e.g.* rate of wilted seedlings, dry weight, and chlorophyll content. Furthermore, roots samples were collected for estimation of Al concentration. Three pots were conducted for each treatment and the experiment was independently repeated three times.

2.8. Quantification of Al in the harvested Korean ginseng roots

Quantification of Al was performed as follows. One g of freeze dried ginseng seedling root was completely grinded, treated with 10 mL HNO_3 and decomposed by the Microwave digestion system (Mars 5, CEM). Then Al was analyzed by the ICP (inductively coupled plasma analyzer, Optima 8400RL, Perkin-Elmer). Standard curve was performed for the quantitative analysis of Al by blotting different define concentration of Al (0, 0.05, 0.1, and 1) mg/L with their corresponded conductivities, then the unknown concentrations of Al in ginseng samples were calculated according to the conductivity obtained.

2.9. Plant growth parameter measurement and statistical analysis

Dry weight of foliar and root parts were separately recorded for ginseng plants harvested from each treatment. Five roots were recorded for each treatment and conducted independently three times. Wilting rate was calculated for both *A. thaliana* and Korean ginseng plants using the following equation [rate of wilted plants = (no. of wilted plants/no. of total plants) × 100]. Chlorophyll contents were extracted from 100 mg of fresh leaves of *A. thaliana* and ginseng seedlings, which were powdered with liquid nitrogen, by 1 mL of 80% cold acetone, and then tubes were incubated in dark for 10 min followed by centrifugation (2500 rpm) for 15 min in 4 °C. Supernatants were collected in new tubes and read in different absorbances 480, 645, 663 nm using spectrophotometer (Arnon, 1949). Three replicates were carried out for each treatment and experiment was independently repeated three times. Relative expression of Al related genes were analyzed by Student's *t* test for the comparison of means ($P < 0.05$). Other experimental data were analyzed via ANOVA followed by Tukey's test ($P < 0.05$) using XLSTAT software 2015.1.

3. Results and discussion

3.1. Molecular identification of the bacteria

Twelve strains were designated and identified based 16S rRNA. Among them, 3 strains belong to the genus *Pseudomonas*, 2 strains belong to the genus *Burkholderia*, 2 strains belong to the genus *Arthrobacter* and others belong to *Leifsonia*, *Bacillus*, *Microbacterium*, *Janthinobacterium*, and *Chryseobacterium* (Table 1).

3.2. In vitro estimation of plant growth promoting traits

For IAA production, the positive results were qualitatively inducted by the red color appearance after adding Salkowski reagent on the broth of each strain and quantitatively determined by spectrophotometer. Most of the strains could not produce IAA in the absence of IAA precursor, L-tryptophan, except *Pseudomonas simiae* N3 (5.5 ± 0.28 mg/L). Majority of the strains could produce IAA in the presence of L-tryptophan. The highest quantity of IAA was detected in the broth inoculated by *Arthrobacter nicotinovorans* N7

Table 1
In vitro plant growth promoting activities of the bacteria isolated from rhizosphere of diseased ginseng roots.

Strain number	Strain Name	Auxins production (mg/L)		Siderophore production[a]	Phosphate solubilization[a]	Growth rate at different mM of Al [b]			
		w $_L$-tryptophan	w/o $_L$-tryptophan			2	4	8	16
N1	*Leifsonia lichenia* 2Sb^T	0	5.7 ± 0.84	−	+	−	−	−	−
N2	*Bacillus aerophilus* 28 K^T	0	0	w	w	−	−	−	−
N3	***Pseudomonas simiae* OLi^T**	**5.5 ± 0.28**	**12.7 ± 1.24**	+	+	+ + + +	+ + +	+	−
N4	*Microbacterium maritypicum* DSM 12512^T	0	7.7 ± 0.51	+	−	+	−	−	−
N5	*Arthrobacter histidinolovorans* DSM 20115^T	0	2.84 ± 97.4	−	−	−	−	−	−
N6	*Pseudomonas extremaustralis* 14-3^T	0	14.0 ± 1.73	−	+	−	−	−	−
N7	*Arthrobacter nicotinovorans* DSM 420^T	0	101.0 ± 17.0	−	w	−	−	−	−
N8	***Pseudomonas fragi* ATCC 4973^T**	**0**	**9.5 ± 0.19**	+	+	+	+	+	−
N9	*Janthinobacterium lividum* DSM 1522^T	0	0	+	−	+	−	−	−
N10	***Chryseobacterium polytrichastri* YG4-6^T**	**0**	**23.3 ± 0.79**	+	−	+ +	+	+	−
N11-1	*Burkholderia ginsengiterrae* DCY85^T	0	0	+	+	+ +	+	+	−
N11-2	***Burkholderia ginsengiterrae* DCY85-1**	**0**	**18.4 ± 1.3**	+	+	+ +	+ +	+	−

Abbreviations; w $_L$-tryptophan, with $_L$-tryptophan; w/o $_L$-tryptophan, without $_L$-tryptophan.
[a] +, positive; w, weakly positive; −, negative.
[b] Range of growth rate (%) (-, < 5; +, 5–20; + +, 20–40; + + +, 40–60; + + + +, 60–80; + + + + +, 80–100).

(101.0 ± 17.0 mg/L), followed by *Chryseobacterium polytrichastri* N10 (23.3 ± 0.79 mg/L), *Burkholderia ginsengiterrae* N11-2 (18.4 ± 1.30 mg/L), *Pseudomonas extremaustralis* N6 (14.0 ± 1.73 mg/L), *Pseudomonas simiae* N3 (12.7 ± 1.24 mg/L), *Pseudomonas fragi* N8 (9.5 ± 0.19 mg/L), *Microbacterium maritypicum* N4 (7.7 ± 0.51 mg/L), *Leifsonia lichenia* N1 (5.7 ± 0.84 mg/L), and finally *Arthrobacter histidinolovorans* N5 (2.84 ± 97.4 mg/L). Other strains did not be able to produce IAA even in the presence of the precursor as shown in Table 1. Production of IAA is variable and depend on many factors, *e.g.* the strain type and the media component. One of the major factor that determine the potentiality of the strain to produce IAA is the addition of the IAA precursors, *e.g.* $_L$-tryptophan (Rajkumar et al., 2005). Therefore, the strain *Pseudomonas simiae* N3 which produced IAA in the absence of $_L$-tryptophan is considered as strong producer of this plant growth regulating hormone, while other strains which produce IAA in the presence of $_L$-tryptophan are considered weak producers.

For siderophores production, the positive result was indicated by the formation of the yellow zone surround the bacterial colony in the blue-green colored media. The results were recorded into two different time points, at 3 and 7 days. The bacteria that showed the positive results after 3 days were recorded as strong producers while those showed the positive results after then were recorded as weak producers. Some strains were recorded as strong producers, others recorded as weak producers and remaining cannot produce siderophores. The strong siderophores producers are *P. simiae* N3, *M. maritypicum* N4, *P. fragi* N8, *Janthinobacterium lividum* N9, *C. polytrichastri* N10, and *B. ginsengiterrae* N11-1, N11-2. Only one strains were recorded as a weak producer, *Bacillus aerophilus* N2 (Table 1). The ability of the bacteria to support the plant growth under heavy metal resistance has been found to be correlated with siderophores production (Siddiqui, 2005; He and Yang, 2007). Therefore, the siderophores producing bacteria in this study are expected to be good candidates for supporting ginseng growth under Al stress.

For phosphate solubilization assay, the positive result was indicated by the development of clear zone surround the bacterial colony in the turbid pikovskaya plates. The determination of strong and weak solubilizers was recorded similarly to the siderophore production. The strong phosphate solubilizers are *L. lichenia* N1, *P. simiae* N3, *Pseudomonas extremaustralis* N6, *P. fragi* N8, *B. ginsengiterrae* N11-1, N11-2 while the weak phosphate solubilizers recorded are *B. aerophilus* N2 and *A. nicotinovorans* N7. Other strains were recorded as non-phosphate solubilizers as indicated by the absence of the clear zone surround the bacterial colony on the turbid pikovskaya plates (Table 1).

3.3. Determination of Al-resistant bacteria

Among the twelve strains tested, seven of them were observed to be Al-resistant as indicated by their ability to grow in Al-immersed NB, however the potentiality of the resistance was found to be varied among those strains; two strains, *M. maritypicum* N4 and *J. lividum* N9 are considered weakly resistant due to their ability to slightly grow in media immersed with only 2 mM of Al while the strains *P. simiae* N3, *P. fragi* N8, *C. polytrichastri* N10, *B. ginsengiterrae* N11-2 and N11-1 are considered highly resistant as was indicated by their ability to grow in Al-containing media broth until the concentration of 8 mM. The highest resistance to Al was recorded for *P. simiae* N3, followed by *B. ginsengiterrae* N11-2, N11-1, *C. polytrichastri* N10, and finally *P. fragi* N8. At the highest concentration of Al added into NB, growth of those strains was completely distorted (Table 1). Metal-resistant *Pseudomonas*, *Burkholderia*, and *Chryseobacterium* species, particularly to Al has been reported previously (Shilpi Mittal and Goel, 2003; Aizawa et al., 2010; Ji et al., 2016). The resistance to Al toxicity was correlated to the production of the siderophores (Shilpi Mittal and Goel, 2003). Most of the Al-resistant strains presented in the current study were also siderophores producers, therefore, we suggested that the tolerance to Al-toxicity is probably due to the siderophores production.

3.4. Expression of Al-related genes of the A. thaliana

Based on the *in vitro* plant growth promoting traits as well as Al-resistance assay, few strains were used for *Arabidopsis* treatment which are stressed by the Al application. The priority of the selection was based on the ability of the strains to tolerate Al as well as their ability to produce siderophores. Accordingly, the strains *P. simiae* N3, *B. ginsengiterrae* N11-2, *P. fragi* N8, and *C. polytrichastri* N10 were selected. In the pot test, it was observed that *Arabidopsis* plants stressed by Al without bacterization, completely wilted and died within 7 days; the plants rosettes were stunted and yellowed compared to mock plants (Fig. 2a) with a wilting rate reached 83.3 ± 5.9% (Table 2). Furthermore, chlorophyll content of negative control plants was significantly declined (Fig. 2b). To confirm the induction of Al toxicity, the pH of the soil was checked before and after applying Al according to the standard methods of the Rural Development Administration, Korea, RDA (1988) and was observed to be drastically reduced (data not shown). On the other hands, plants treated with the selected bacteria before Al stress application, showed different morphological responses; Al-stressed plants treated with the strains *P. simiae* N3, *B. ginsengiterrae* N11-2, and *C. polytrichastri* N10 have similar appearance of the mock plants especially those treated with *P. simiae* N3 (Fig. 2a) with only

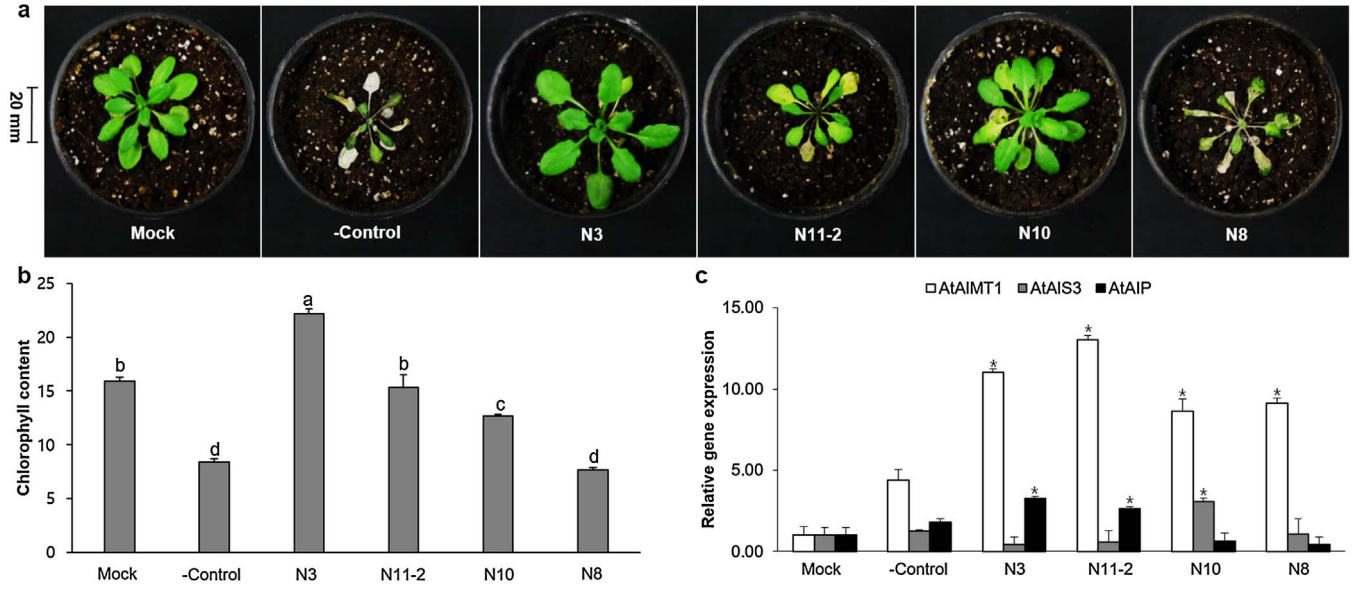

Fig. 2. *Arapidopsis thaliana* plants inoculated or not with candidate PGPB, *Pseudomonas simiae* N3, *Pseudomonas fragi* N8, *Chryseobacterium polytrichastri* N10, and *Burkholderia ginsengiterrae* N11-2 after 7 days from the application of 500 mM of $Al_2(SO_4)_3$. **a**, Morphological differences; **b,** Chlorophyll content and **c**, The quantitiative analysis of Al-stress related genes, *AtALMT1*, *AtALS3*, and *AtAIP*. Values are expressed as mean ± SE. Results of chlorophyll content are statistically analyzed via ANOVA, data columns with the same letter are not significantly different ($P < 0.05$, Tukey's test, $n = 3$). Results of gene expression are statistically analysed via Student's *t* test, * indicate significant difference ($P < 0.05$, n = 3). Abbreviations; PGPB, plant growth promoting bacteria; Al, Aluminium; *AtALMT1*, Aluminium-activated maleate trasporter 1 gene of *Arapidopsis thaliana*; *AtALS3*, Aluminium sensitive 3 gene of *Arapidopsis thaliana*; *AtAIP*; Aluminium induced protein gene of *Arapidopsis thaliana*.

Table 2
The rate of wilted *Arabidopsis thaliana* plants treated or not with PGPB and then stressed by 500 mM of $Al_2(SO_4)_3$.

Treatment	Wilting rate (%)
Mock	0[c]
− Control	83.3 ± 5.9[a]
P. simiae N3	27.8 ± 9.0[bc]
B. ginsengiterrae N11-2	36.1 ± 3.4[b]
C. polytrichastri N10	47.2 ± 6.8[b]
P. fragi N8	80.5 ± 9.0[a]

Values are expressed as mean ± SE. Data with the same letter are not significantly different ($P < 0.05$, Tukey's test, $n = 3$). Abbreviation; PGPB, plant growth promoting bacteria.

Table 3
qRT-PCR primers used for determination of transcript levels of Al-induced related genes as well as housekeeping gene (*Actin*) after application of 500 mM of $Al_2(SO_4)_3$ on *Arabidopsis thaliana* treated or not with PGPB.

Primer	Sequence (5′–3′)	Tm (°C)
AtAIP-F	TCGTCGAGAAAAATCCATCC	52.4
AtAIP-R	CAATGGAACCTTGCCAACTT	53.8
AtALS3-F	TGTTTCCCGATCGTTTCTTC	52.8
AtALS3-R	TAATCCGGCCACGTACTTTC	54.7
AtALMT1-F	GAGAGCTCGGTGAAAAGGTG	55.7
AtALMT1-R	CGTGGTTTTCTGGTGGATCT	55.0
AtActin2-F	GTGTGTCTTGTCTTATCTGGTTCG	55.5
AtActin2-R	AATAGCTGCATTGTCACCCGATAC	57.2

Abbreviation; qRT, quantitative real time; Al, Aluminum; PGPB, plant growth promoting bacteria; *AtAIP*, Aluminum induced protein gene of *Arabidopsis thaliana*; *AtALS3*; Aluminum sensitive 3 gene of *Arabidopsis thaliana*; *AtALMT1*; Aluminum-activated maleate transporter 1 gene of *Arabidopsis thaliana*.

27.8 ± 9.0% wilting rate (Table 2), however leaves were found to acquire significantly higher chlorophyll content (Fig. 2b). The inoculation of radish plants by *Pseudomonas fluorescens* under salinity stress lead to the increase of the chlorophyll content, compared to non-inoculated as well as mock plants (Mohamed and Gomaa, 2012), however the reason for this observation is still unclear. Other plants treated with *B. ginsengiterrae* N11-2, and *C. polytrichastri* N10 showed reduced growth and limited yellowing on the rosettes area (Fig. 2a) with wilting rate reached 36.1 ± 3.4% and 47.2 ± 6.8%, respectively (Table 2). The chlorophyll content of the plants' leaves treated with these two bacteria were significantly reduced compared to those of mock plants, however the content still significantly higher than those of negative control plants (Fig. 2b). Only the group of plants treated with *P. fragi* N8 were not survived and completely wilted, similarly to the negative control plants (Fig. 2a, b; Table 2) which give an evidence that the production of siderophores by the treated bacteria is not the sole mechanism by which other bacteria protect the plant from Al toxicity.

qRT-PCR analysis showed differential expression pattern of Al-tolerant related genes, which matched with the morphological observations; *Arabidopsis* plants stressed by Al without bacterization showed slightly high expression of *AtALMT1* and *AtAIP*, however there was no significant difference compared to mock plants (Fig. 2c). In the study which utilized the characterization of *AIP* from Korean ginseng, expression of *ALMT1* and tolerance against Al stress in the wild type *Arabidopsis* plants was associated with higher expression of *AIP* in *A. thaliana* (Jang et al., 2014). According to this finding along with the presented results, it is postulated that *AIP* might act as a regulator for expression of *ALMT1* and induction of external mechanism of Al tolerance, however the expression of those genes in the present data was slightly increased in the negative control plants, possibly because of the high concentration of the applied Al. In case of the bacterized plants, significant differences were detected in the expression of the Al-tolerant related genes, but the expression profile of the genes changed among the plants groups depend on the inoculated bacteria. For example, the plants treated with *P. simiae* N3 and *B. ginsengiterrae* N11-2 showed significantly high expression rate of *AtALMT1* and *AtAIP* compared to those of mock plants; *AtALMT1* was eleven and thirteen fold increased, respectively and *AtAIP* was four and three fold increased, respectively. Furthermore, the expression of *AtALS3* was observed to be reduced, but not significantly, in plants treated by both bacteria (Fig. 2c). These results indicate that the inoculation of *P. simiae* N3 and *B. ginsengiterrae* N11-2 lead to the upregulation of Al- stress related genes controlling only the external tolerance mechanism. Expression profile of the genes in plants bacterized by *C. polytrichastri*

Fig. 3. Morphological differences 2-year old of Korean ginseng seedlings inoculated or not with candidate PGPB, *Pseudomonas simiae* N3, *Chryseobacterium polytrichastri* N10, and *Burkholderia ginsengiterrae* N11-2 after 7 days from the application of 500 mM of $Al_2 (SO_4)_3$. Abbreviations; PGPB, plant growth promoting bacteria.

Table 4
Effect of PGPB treatment on Korean ginseng growth and Al accumulation in the roots after application of 500 mM of $Al_2 (SO_4)_3$ solution.

Treatment	Wilting rate (%)	Ginseng dry weight (mg)		Detected Al (μg/g)
		Shoot	Root	
Mock	0^{c}	$110n \pm 0.8^{a}$	210 ± 8.5^{a}	957.3 ± 36.78^{c}
−control	51.1 ± 5.4^{a}	80 ± 1.6^{b}	180 ± 8.6^{a}	1974.7 ± 154.54^{a}
P. simiae N3	17.8 ± 2.7^{bc}	100 ± 8.6^{a}	200 ± 1.6^{a}	1367.6 ± 38.61^{b}
B. ginsengiterrae N11-2	22.2 ± 2.7^{b}	120 ± 3.7^{a}	210 ± 3.7^{a}	1149.2 ± 99.09^{bc}
C. polytrichastri N10	28.9 ± 7.2^{b}	110 ± 3.5^{a}	200 ± 7.5^{a}	2051.5 ± 126.15^{a}

Values are expressed as mean ± SE. Data with the same letter are not significantly different ($P < 0.05$, Tukey's test, $n = 3$). Abbreviation; PGPB, plant growth promoting bacteria; Al, Aluminum.

N10 was different; the expression of *AtALMT1* was also significantly high, but the level of the expression was lower than those of *P. simiae* N3 and *B. ginsengiterrae* N11-2 bacterized plants (only nine fold increased compared to mock plants). Furthermore, the expression of *AtAIP* gene was reduced, but not significantly. However, *AtALS3* was observed to be significantly increased compared to *P. simiae* N3 and *B. ginsengiterrae* N11-2 bacterized plants as well as mock plants (Fig. 2c). This expression profile indicates that the application of *C. polytrichastri* N10 lead to the induction of the internal mechanism of the Al tolerance. It has been reported that the plant model pathogen, *Pseudomonas syringae* induce *ALMT1* in *Arabidopsis* and tomato plants (Lakshmanan et al., 2012; Ding et al., 2013; Lakshmanan and Bais, 2013). Furthermore, experimental analysis showed that one of the factor causes induction of *Arabidopsis* plants' growth under Al stress by the member of *Paenibacillus* and *Micrococcus* is the induction of Al-stress related genes, *AtALMT*, *AtAIP,* and *AtALS3* (Sukweenadhi et al., 2015). According to our knowledge, the data in the present study report, for the first time, the induction of Al-stress related genes in *Arabidopsis* by *P. simiae* and members of *Burkholderia* and *Chryseobacterium*. On the other hand, plants treated with *P. fragi* N8 showed significantly high expression rate of only *AtALMT1* (which was nine fold increase compared to mock plants), and low expression rate of the genes of *AtAIP* and *AtALS3*. The presented results showed induction of *AtALMT1* expression, however the plants failed to survived under the stress of Al. This observation suggested more that the induction of successful external tolerance mechanism against Al requires the induction of both *AtALMT1* and *AtAIP* genes.

3.5. Ginseng plant stress and Al quantification

The candidate strains which succeed to support *Arabidopsis* plants under Al stress were selected for further assay on Korean ginseng seedlings. Two-year old fully sprouted ginseng seedlings were one time irrigated by the selected strains, *P. simiae* N3, *B. ginsengiterrae* N11-2, and *C. polytrichastri* N10, 10 days prior to the Al stress application. After Al application, the morphology of the bacterized seedlings was

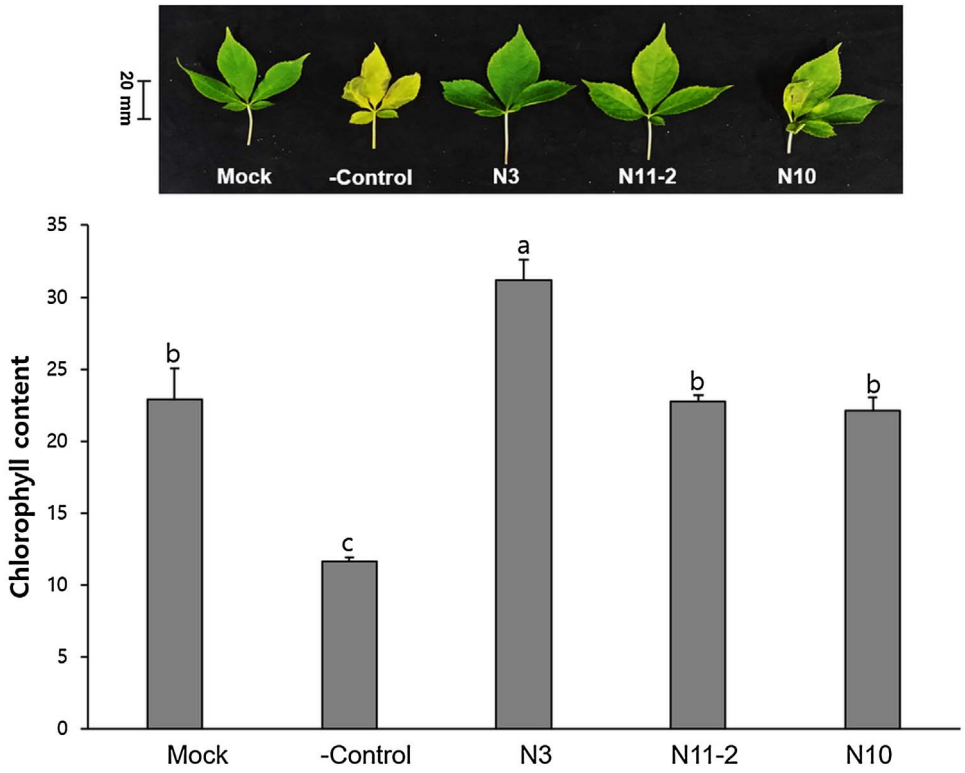

Fig. 4. Chlorophyll content of 2-year old of Korean ginseng seedlings' leaves inoculated or not with candidate PGPB, *Pseudomonas simiae* N3, *Chryseobacterium polytrichastri* N10, and *Burkholderia ginsengiterrae* N11-2 after 7 days from the application of 500 mM of $Al_2(SO_4)_3$. Values are expressed as mean ± SE. Data columns with the same letter are not significantly different ($P < 0.05$, Tukey's test, $n = 3$). Abbreviations; PGPB, plant growth promoting bacteria.

monitored in comparison with Al stressed, non-bacterized seedlings (negative control) as well as mock seedlings for 7 days. In negative control seedlings, yellowing symptom was gradually developed on the leaves part which ended by the completely wilting of the foliage while leaves of mock seedlings were remaining green at the same period of time (Fig. 3). The wilting rate of the negative control seedlings was found to be significantly high (Table 4). Sequentially, chlorophyll content and dry weight of the negative control seedlings' foliage were found to be significantly declined (Fig. 4; Table 4). Furthermore, roots of negative control seedlings were observed to be morphologically different; the number and the length of the fine roots was reduced compared to those of mock seedlings' roots. In addition, the direction of the tip part was shifted upward giving the v-shaped appearance to the root part (Fig. 3), however the dry weight does not be significantly reduced (Table 4). The pH of the ginseng soil was checked before and after Al application as performed with *Arabidopsis* soil and was found to be highly decreased as well (data are not shown). Based on these observations, it is postulated that the applied Al retards the growth of fine roots which in turn lead to the reduction of the nutrient uptake and finally cause reduction of foliage growth. On the other hand, the leaves of Al stressed, bacterized seedlings were morphologically similar to the mock seedlings (Fig. 3). Also, the wilting rate was significantly reduced compared to negative control seedlings (Table 4). Chlorophyll content as well as the dry weight of the foliage of the bacterized seedlings did not be significantly changed, except for those of seedlings treated with *P. simiae* N3; chlorophyll content of the foliage was significantly increased while the dry weight did not be significantly increased (Fig. 4; Table 4). Furthermore, the growth and morphology of roots of the bacterized seedlings did not be significantly changed; the fine root numbers and length, the direction of the root tip and the dry weight of the roots were explicitly similar to those of the mock seedlings (Fig. 3; Table 4). All these observations indicate the induction of tolerance response against the applied Al due to the bacterial inoculation.

Quantity of Al in the roots of mock and negative control seedlings were 957.3 ± 36.78 μg/g and 1974.7 ± 154.54 μg/g, respectively. The quantity of Al in roots inoculated with *P. simiae* N3, *B. ginsengiterrae* N11-2 was significantly reduced (1367.6 ± 38.61 and 1149.2 ± 99.09 μg/g) while increased, but not significant, in roots inoculated with the strain *C. polytrichastri* N10 (2051.5 ± 126.15 μg/g) compared to negative control seedlings' roots (Table 4). Based on Al analysis, it is assumed that the inoculation of the strains *P. simiae* N3, *B. ginsengiterrae* N11-2 induces the external mechanism of tolerance while the inoculation of *C. polytrichastri* N10 strain induces the internal mechanism of tolerance in ginseng seedlings similarly to *Arabidopsis*.

4. Conclusion

Out of 12 strains isolated from rhizosphere of diseased Korean ginseng roots located in Gochang province, Republic of Korea, 4 strains were chosen according to their ability to tolerate Al and their plant growth promoting activities, particularly siderophores production for *Arabidopsis* treatment under Al stress. Three strains only, *P. simiae* N3, *B. ginsengiterrae* N11-2, and *C. polytrichastri* N10 could be able to support *Arapidopsis* growth under Al stress which is supported by upregulation of Al-stress related genes. *P. simiae* N3, *B. ginsengiterrae* N11-2, and *C. polytrichastri* N10 were also able to support Korean ginseng seedlings growth under Al stress. The expression profile of *Arabidopsis* Al-stress related genes as well as Al analysis in treated ginseng seedlings' roots suggest that the inoculation of *P. simiae* N3 and *B. ginsengiterrae* N11-2 might induce the external mechanism of Al tolerance while the inoculation of *C. polytrichastri* N10 lead to the induction of internal mechanism of Al tolerance in ginseng seedlings. According to the presented data, the strains *P. simiae* N3, *B. ginsengiterrae* N11-2, and *C. polytrichastri* N10 can be used in the future for the applicable approach as inoculants to support the ginseng crop in Al-contaminated soil and accordingly reduce the range of crop loss.

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