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Isolation and characterization of plant growth promoting endophytic diazotrophic bacteria from Korean rice cultivars☆

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ABSTRACT

We have isolated 576 endophytic bacteria from the leaves, stems, and roots of 10 rice cultivars and identified 12 of them as diazotrophic bacteria using a specific primer set of *nif* gene. Through 16S rDNA sequence analysis, *nifH* genes were confirmed in the two species of *Penibacillus*, three species of *Microbacterium*, three *Bacillus* species, and four species of *Klebsiella*. Rice seeds treated with these plant growth-promoting bacteria (PGPB) showed improved plant growth, increased height and dry weight and antagonistic effects against fungal pathogens. In addition, auxin and siderophore producing ability, and phosphate solubilizing activity were studied for the possible mechanisms of plant growth promotion. Among 12 isolates tested, 10 strains have shown higher auxin producing activity, 6 isolates were confirmed as strains with high siderophore producing activity while 4 isolates turned out to have high phosphate-solubilizing activity. These results strongly suggest that the endophytic diazotrophic bacteria characterized in this study could be successfully used to promote plant growth and inducing fungal resistance in plants.

1. Introduction

Soil is replete with microscopic life forms including bacteria, fungi, nematodes, and algae. Over 95% of the bacteria exist in the plant roots and those plants obtain many nutrients through the soil bacteria. Nitrogen is used to synthesize plant proteins and nucleic acids, including DNA. Although, it is found naturally in the atmosphere, it cannot be used by the plants in the available form (N_2). Nitrogen can be combined chemically with oxygen or hydrogen to form various nitrogenous compounds that plants can use. These nitrogenous compounds can then be added to the soil in the form of ammonium (NH_4^+) and nitrate (NO_3^+) fertilizer. Especially, the use of nitrogen fertilizer is of great importance in rice production, as nitrogen is the major factor limiting growth under most conditions (Dawe et al. 2000). Thus, N_2 fixation is a prime requisite for plant growth particularly in crops like rice. N_2 fixers, also called 'diazotrophs' play a critical role in the plant ecosystem by reducing dinitrogen (N_2) to ammonia (NH_3) (Dilworth 1974). Previous reports have indicated that diazotrophs show ameliorating effects on nutrient uptake, stress tolerance, phytohormone and vitamin synthesis, inorganic phosphate solubilization and overall plant growth promotion (Sachdev et al. 2009; Martinez-Viveros et al. 2010; Ali et al. 2010; Bhattacharyya and Jha 2012; Gururani et al. 2012). In addition, diazotrophs are known to counter the detrimental effects that follow with the onset of a pathogen attack. Previous reports suggest that diazotrophs are capable of synthesizing antibiotics; anti-fungal compounds *etc.*, *via* competing for nutrients by producing siderophores or by triggering the induced systemic resistance (ISR) against pathogens (Dobbelaere et al. 2003).

After nitrogen (N), phosphorus (P) is the most limiting nutrient for crop yields, and is particularly essential for rice growth and development. Phosphorus-deficient plants usually show inhibited stem and root development, poor flowering, lack of seed and fruit formation *etc.*, consequently causing degradation in quality and quantity. Notably, the production of nitrogen fertilizers depends on the use of non-renewable resources such as oil, gas, or coal (Stoltzfus et al. 1997). In recent years, the use of bio-inoculants composed of diazotrophic bacteria as an alternative to nitrogen fertilizers (Welbaum et al. 2004) has emerged as a promising approach. Nitrogen-fixing bacteria belonging to PGPB (Plant Growth Promoting Bacteria) can fix atmospheric nitrogen and supply it to plants. Here we use the term PGPB as bacteria including diazotrophic bacteria or plant growth-promoting rhizobacteria (PGPR). PGPB can competitively colonize plant root, promote plant growth, and reduce plant diseases. Plant growth-promoting rhizobacteria genera: *Bacillus* (Idriss et al. 2002), *Enterobacter* (Gupta et al. 1998) and *Corynebacterium* (El-Banana and Winkelmann 1988) have been reported to benefit plants by enhancing plant

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growth and improving plant health through various direct and indirect mechanisms. PGPB are commonly used as inoculants for improving the growth and yield of agricultural crops and offers an attractive way to replace chemical fertilizers, pesticides, and supplements (Ştefan et al. 2008; Ashrafuzzaman et al. 2009; Saharan and Nehra 2011). These bacteria significantly affect plant growth by increasing nutrient uptake, producing biologically active phytohormones and suppressing pathogens by producing antibiotics, siderophores, and fungal cell wall-lysing enzymes (Arora et al. 2001; Persello-Cartieaux et al. 2003; Kuklinsky-Sobral et al. 2004; Frey-Klett et al. 2005; Hameeda et al. 2008). Among these, auxin is one of the most vital hormones, primarily due to its pivotal functions in the initial processes of lateral and adventitious root formation (Gaspar et al. 1996; Idris et al. 2007) and root elongation (Yang et al. 1993). Earlier reports indicate that PGPB may also enhance plant auxin synthesis (Kloepper et al. 2004; Yao et al. 2006). PGPB are known to release metal-chelating substances such as iron-chelating siderophores into the rhizosphere. These siderophore-producing PGPB then influence the uptake by plants of various metals, including Fe, Zn, and Cu (Carrillo-Castaneda et al. 2005; Egamberdiyeva 2007; Dimkpa et al. 2008, 2009; Gururani et al. 2012). Besides, siderophores as determinants of induced systemic resistance also play an important role in protection of crops from plant pathogens (Ramamoorthy et al. 2001; Kirankumar et al. 2008).

PGPB could also promote plant growth by suppressing plant pathogens indirectly. This enhanced state of resistance is effective against a broad range of pathogens and parasites, including fungi, bacteria, viruses, nematodes, parasitic plants, and insects (Vauterin and Swings 1997; Murphy et al. 2003; Ryu et al. 2004). In the last few years, the number of PGPB that have been identified has seen a great increase. Species of bacteria like *Pseudomonas*, *Azospirillum*, *Azotobacter*, *Klebsiella*, *Enterobacter*, *Alcaligenes*, *Arthrobacter*, *Burkholderia*, *Bacillus* and *Serratia* have been reported to enhance the plant growth (Kloepper et al. 1989; Okon and Labandera-Gonzalez 1994; Glick 1995; Gururani et al. 2012). In a previous report, a nitrogen fixing bacteria, *Bacillus megaterium* was isolated from maize rhizosphere which did not show any antifungal activity (Liu et al. 2006). In another recent report, *Bacillus subtilis* was isolated from roots of banana plant and it was concluded that although, *B. subtilis* is not a nitrogen fixing bacterium, it can be efficiently used in a bio-organic fertilizer against *Fusarium* wilt (Zhang et al. 2011). To the best of our knowledge, there are no such reports available yet, where nitrogen fixing endophytic bacteria from rice root has shown both growth promotion as well as antifungal activity.

In the present study, endopytic diazotrophic bacteria were isolated from various rice cultivars in Korea. The isolates were then identified and characterized for their functional traits associated with plant growth promotion and induced systemic resistance.

2. Materials and methods

2.1. Microorganisms and growth conditions

For nitrogen fixing bacteria (NFB), Tryptic soy broth (TSB, Tryptic soy broth 3%; Difco, USA) and nitrogen-free semi-solid media agar plates were used (malic acid 4 g, KH_2PO_4 0.48 g, $MgSO_4{\cdot}7H_2O$ 0.16 g, NaCl 0.08 g, $CaCl_2$ 0.016 g, $FeCl_3$ 0.008 g, $Na_2MoO_4{\cdot}2H_2O$ 0.0013 g, H_3BO_3 0.00224 g, Cu $(NO_3)\cdot$ $3H_2O$ 0.00093 mg, $ZnSO_4{\cdot}7H_2O$ 0.192 mg, morpholinoethane sulfonic acid (MES) 20 mM per liter (pH 6.8). The NFB was grown on TSA agar or in TSB broth medium at 30 °C for 2days. All isolated strains were stored at −70 °C in TSB broth containing 15% (v/v) glycerol.

2.2. Rice sample preparation and isolation of nitrogen-fixing bacteria

Leaf, stem and root samples of various rice cultivars (*Oryza sativa* var. *Japonica* c.v. Chilbo, Chuchung, Haiami, Ilpum, Migwang, Nampyung, Sechuchung, Wongwang, Hwayung) were collected for isolating nitrogen-fixing bacteria. The endophytic diazotrophic microorganisms from roots, stems, and leaves of rice were isolated using nitrogen free semi-solid media. Plant tissue samples were surface sterilized with 70% ethanol for 1 min and shaken in 1.2% (w/v) NaClO solution for 15 min. Samples were then washed three times with sterile distilled water with shaking (15 min each). Surface sterilized samples were ground with sterilized mortar and pestle, and inoculated on nitrogen free semi-solid agar media. After incubation at 30 °C for 2 days, the inoculants were transferred to fresh nitrogen free media and then incubated at 30 °C for 2 days. The transfer procedure mentioned above was carried out 3–4 times to isolate single colonies (Choi et al. 2003; Lee et al. 2005). Nitrogen fixing bacteria were stored at −70 °C in TSB broth containing 15% (v/v) glycerol.

2.3. Partial identification of nitrogen-fixing bacteria by 16S rDNA sequence analysis

2.3.1. DNA extraction

Rice plants selected at the heading stage were dug out from a wetland rice field, and ground to a fine powder in a mortar and pestle. The fine powder was suspended in extraction buffer (100 mM Tris, 100 mM EDTA, 250 mM NaCl, 100 μg of proteinase K per ml) supplemented with sarkosyl (1% final concentration) and lysed by incubation at 55 °C for 1 h. Treatment of the lysate with RNase A was followed by chloroform extraction and isopropanol precipitation. Crude DNA was purified by phenol extraction, chloroform extraction, and isopropanol precipitation.

2.3.2. PCR amplification of nifH genes and 16S rDNA sequence analysis

The nitrogenase iron protein gene *nifH* is one of the oldest existing and functioning genes in the history of gene evolution, and the outline of the *NifH* tree is reported to be largely consistent with the 16S rDNA phylogeny (Young 1992, 1993). So, the existence of *the nifH gene* could be an indirect evidence for nitrogenase activity of some bacteria. Since *nif* genes are conserved among a broad spectrum of bacteria, the use of universal primers has enabled the amplification and analysis of *nifH* sequences from various microorganisms and environmental samples. The primers for PCR amplification used were: 19F (5′′-GCIWTYTAYGGIAARGGIGG-3′) and 407R (5′-AAICCRCCRCAIACIACRTC-3′). PCR fragments (390 bp) of *nifH* were amplified between nucleotides 19 and 407 (*Azotobacter vinelandii* M20568 munbering) from rice leaf, stem and root DNA. The PCR reaction conditions with 100 ng of template DNA were: 1 min at 94 °C, 1 min at 50 °C, and 50 s at 72 °C for 30 cycles. Sterile milliQ water was used as negative controls. Bacterial isolates were partially identified by the analysis of 16S rDNA sequence. Specific primers used for PCR were: fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and rp2 (5′-ACGGCTACCTTGTTACGACTT-3′) (Weisburg et al. 1991). PCR mixture contained 50 ng of template DNA, primers of 10 pmol concentration, PCR master mix containing Taq DNA polymerase, dNTPs, Tris–HCl, $MgCl_2$ stabilizer, and tracking dye were used according to the manufacturer's instructions (TaKaRa bio Inc., Japan). The reaction conditions were, 1 min at 94 °C, 1 min at 55 °C, and 1 min 50 sec at 72 °C for 30 cycles. The expected size of amplicon was 1.4 kb. The amplified fragments were recovered from agarose gel using a gel extraction kit (Solgent, Korea). The nucleotide sequences were then determined through 16S rDNA gene sequencing (Macrogen, Korea). The 16S rDNA gene sequences