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Isolation and Characterization of Plant Growth-Promoting Rhizobacteria from Wheat Roots by Wheat Germ Agglutinin Labeled with Fluorescein Isothiocyanate

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Thirty-two isolates were obtained from wheat rhizosphere by wheat germ agglutinin (WGA) labeled with fluorescein isothiocyanate (FITC). Most isolates were able to produce indole acetic acid (65.6%) and siderophores (59.3%), as well as exhibited phosphate solubilization (96.8%). Fourteen isolates displayed three plant growth-promoting traits. Among these strains, two phosphate-dissolving ones, WS29 and WS31, were evaluated for their beneficial effects on the early growth of wheat (*Triticum aestivum* Wan33). Strain WS29 and WS31 significantly promoted the development of lateral roots by 34.9% and 27.6%, as well as increased the root dry weight by 25.0% and 25.6%, respectively, compared to those of the control. Based on 16S rRNA gene sequence comparisons and phylogenetic positions, both isolates were determined to belong to the genus *Bacillus*. The proportion of isolates showing the properties of plant growth-promoting rhizobacteria (PGPR) was higher than in previous reports. The efficiency of the isolation of PGPR strains was also greatly increased by WGA labeled with FITC. The present study indicated that WGA could be used as an effective tool for isolating PGPR strains with high affinity to host plants from wheat roots. The proposed approach could facilitate research on biofertilizers or biocontrol agents.

Keywords: PGPR, FITC-labeled WGA, *Bacillus* sp.

Introduction

Plant growth-promoting rhizobacteria (PGPR) are a group of soil microorganisms that can stimulate the growth of plants, protect them from diseases, and increase their yield (Germida *et al.*, 1998). PGPR significantly promote plant growth by increasing nutrient availability and suppressing pathogens in two ways in general. First is by producing phytohormones such as auxins and other plant hormones (cytokinin and gibberellin). Second is by producing antibiotics, siderophores, or bacterial and fungal antagonistic substances (Glick, 1995; Senthilkumar *et al.*, 2007). However, many isolated strains often fail to induce desired effects when they are applied in the field. The failure is due to their inability to compete with the native microflora, or hindrance by soil types and climatic conditions (Bashan, 1998; Johnsson *et al.*, 1998). Hence, an effective method for isolating PGPR is needed. Khalid *et al.* (2004) have used auxin as an indicator to screen effective PGPR that can be applied in the field from the rhizosphere soil of wheat plants. Abbasi *et al.* (2011) have also screened PGPR from wheat rhizosphere to evaluate their potential use in plant growth improvement by phytohormone production. Fischer *et al.* (2007) have isolated native strains from the rhizosphere and endorhizosphere of wheat according to their nitrogen-fixing abilities. One siderospore-producing strain, *Bacillus subtilis* CAS15, exhibits a growth-promoting effect on pepper plants (Yu *et al.*, 2011). However, their work has mainly focused on bacteria with abilities to produce phytohormones and siderophores, or fix nitrogen. Such methods are believed to be valuable in isolating PGPR from rhizosphere. Nevertheless, little attention has been paid to the isolation of rhizobacteria possessing specific relationships with plants. Consequently, a new technique using lectin as a tool to screen crop-specific bacteria from wheat roots was designed in the present study.

Lectins are proteins that reversibly and nonenzymatically bind to specific carbohydrates (Pistole, 1981; Peumans and Damme, 1995). Lectins, which are widely found in the cells of plants, animals, and microbes (De Hoff *et al.*, 2009), play important roles in recognition or resistance in hosts and microbes (Moreira *et al.*, 1991; Peumans and Damme, 1995; Nathan, 2008; De Hoff *et al.*, 2009). Plant lectins are found in many plant species, such as wheat (Aub *et al.*, 1965; Burger and Goldberg, 1967), jack bean (Inbar and Sachs, 1969), and soybean (Sela *et al.*, 1970). Wheat germ agglutinin (WGA), one of the most widely studied grass lectins, is secreted and exposed at the root surface. The majority of WGAs is distributed on the surface of young roots (Mishkind *et al.*, 1980). WGA could be a specific attachment site for the putative receptors (e.g., capsular glycoproteins, polysaccharides, and flagella) of bacteria, and could direct bacteria adherence to the root surface (Del Gallo and Fendrik, 1994; Antonyuk and Evseeva, 2006). Antonyuk and Ignatov (2001) have reported that WGA can act as a signal

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molecule to stimulate bacterial metabolism that benefits host plants. Hence, WGA has great importance in plant-microbial interactions. Considering all these conditions, WGA was used in the present study as a tool for isolating effective and crop-specific bacteria from wheat plant rhizosphere.

The isolation of bacteria from plant rhizospheres has been extensively studied (Khalid *et al.*, 2004; Ding *et al.*, 2005; Park *et al.*, 2005; Flores-Vargas and O'Hara, 2006; Fischer *et al.*, 2007; Senthilkumar *et al.*, 2007; Kumar *et al.*, 2010; Abbasi *et al.*, 2011). However, little attention has been paid to the ability of lectins in recognizing plants and rhizobacteria. Some of the present authors have previously revealed the existence of a specific affinity between WGA and wheat rhizobacteria. The objective of the present study was to isolate PGPR strains from wheat plant roots using WGA labeled with fluorescein isothiocyanate (WGA-FITC).

Materials and Methods

Soil sample collection and PGPR isolation

Wheat rhizosphere soil samples were carefully collected from the cities of Fuyang (115.82° E and 32.9° N) and Hefei (117.27° E and 31.85° N) in Anhui province. All samples were kept in sterilized paper bags and stored at 4°C. About 10 g of rhizosphere soil was transferred to a 250-ml Erlenmeyer flask containing 100 ml of sterile distilled water, and the flask was shaken at 150 rpm for 30 min. Serial dilutions were immediately prepared, and 0.1 ml aliquots (10^{-6} to 10^{-3}) were spread on selective solid medium plates. Three media were used. First was a modified Ashby medium (pH 7.0 to 7.2), which comprised (in g/L): glucose, 10.0 KH_2PO_4, 0.2 $MgSO_4{\cdot}7H_2O$, 0.2 NaCl, 0.2 $Ca_2SO_4{\cdot}2H_2O$, 0.1 $CaCO_3$, 5.0 and agar, 15.0 to 20.0 (Olyunina *et al.*, 2009). Second was a modified Pikovskaya medium (pH 7.0 to 7.5) comprising (in g/L): NaCl, 0.3; $MgSO_4{\cdot}7H_2O$, 0.3; $MnSO_4{\cdot}4H_2O$, 0.03; KCl, 0.3; $(NH_4)_2SO_4$, 0.5; $FeSO_4{\cdot}7H_2O$, 0.03; $Ca_3(PO_4)_2$, 5.0; sucrose, 10.0; and agar, 15.0 to 20.0 (Alagawadi and Gaur, 1988). Third was a potassium-releasing bacteria medium (pH 7.0 to 7.5) comprising (in g/L): Na_2HPO_4, 2.0; $MgSO_4{\cdot}7H_2O$, 0.5; $FeCl_3$, 0.05; sucrose, 5.0; $CaCO_3$, 0.1; glass powder, 1.0; and agar, 15.0 to 20.0.

The plates were incubated for 5 d at 28°C. Morphologically different colonies appearing on the medium were isolated and purified by streaking on plates with the same medium. All purified isolates were stored at -20°C until analyses.

Rescreening of isolates by staining with WGA-FITC

WGA was purified by precipitation with ammonium sulfate and chitin affinitive chromatography according to the method described by Levine *et al.* (1972). Labeling with FITC was performed per the method described by Bohlool and Schmidt (1974). The WGA-FITC (2 mg/ml) was stored at -20°C.

The isolated strains were collected from plates, spread in one drop of distilled water on a slide glass, and air dried. About 25 µl of WGA-FITC solution was immediately added to the slide, which wasincubated for 30 min at 25°C. The fixed cells were rinsed with 10 mM/L phosphate-buffered saline (pH 7.2) and distilled water for 15 min and 1 min, respectively. The slide was then air dried for 2 h in a dark room. Visualization was performed using a fluorescent microscope with an FITC filter (maximum excitation = 490 nm and maximum emission = 520 nm green fluorescence Leica DMLB, Germany). Isolates initially incubated in WGA solution (2 mg/ml not labeled with FITC), rinsed, and air dried as the above procedure served as controls. Isolates that could be stained by WGA-FITC but not by pre-incubated WGA solution were considered positive, and were stored for further analysis.

Phenotypic characterization

Physiological and biochemical characteristics of the bacterial isolates that could be stained by WGA-FITC were examined according to the methods described in Bergey's Manual of Determinative Bacteriology Edition 8.0 (Holt *et al.*, 1994). All strains were characterized by Gram staining and light microscopy. Such traits as colony morphology, endospore, catalase, and starch hydrolysis were characterized. Plate assays were employed to determine the carbon source utilization.

Characterization of plant growth-promoting traits

Indole acetic acid (IAA) production was measured by the colorimetric method (Gordon and Weber, 1951). The isolates were cultivated in a minimal medium (Park *et al.*, 2011) at 25°C for 7 d in a shaking incubator at 180 rpm.

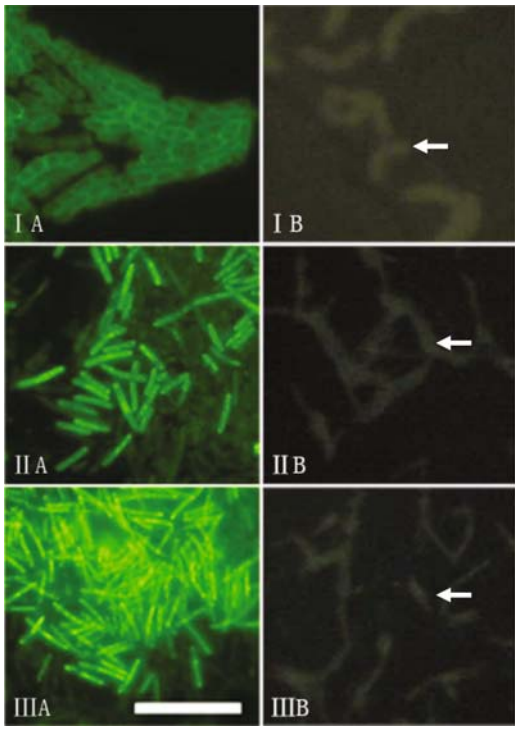

Fig. 1. Fluorescent photos of isolates stained by WGA-FITC. (IA), (IIA), and (IIIA) are strain WS24, WS31, and WS32, respectively. (IB), (IIB), and (IIIB) are control treatment of WS24, WS31, and WS32, respectively. Arrows mark the no-stained cells. Bar represents 10 µm.

Bacterial cells were removed from the culture broth by centrifugation (1.5 ml of bacterial suspension). Supernatants were vigorously mixed at a 1:2 ratio with Salkowski's reagent, and incubated in the dark for 30 min at 25°C. The absorbance of the final solution was measured at 530 nm. The concentration of IAA in the culture medium was determined using the standard curve of pure IAA (Sangon Biotech Co., Ltd., China).

Siderophore production was determined using an Fe-deficient mineral salt medium (MSM) (Park *et al.*, 2011). The selected strains were inoculated in the MSM and incubated in a shaking incubator at 25°C for 3 d at 180 rpm. The cell-free culture supernatants were assayed for siderophore production using the Chrome Azurol S assay (Schwyn and Neilands, 1987).

Phosphorus solubilization by selected PGPR strains was quantified using insoluble tricalcium phosphate in modified Pikovskaya medium. About 1 ml of bacterial suspension was placed in a 250 ml Erlenmeyer flask containing 100 ml of the medium, and incubated for 7 d at 25°C. The culture medium was centrifuged to remove bacteria cells, and the suspension was used to analyze phosphorus concentration. Solubilized phosphorus was quantified according to the phospho-molybdate blue color method (Gull *et al.*, 2004).

Sequencing of 16S rDNA gene

Most of the isolates showing PGPR properties were identified based on 16S rDNA sequencing. Bacterial genomic DNA was extracted and purified using a Sangon Bacterial Genomic DNA Extraction UNIQ-10 kit according to the

Table 1. Selected physiological and biochemical characteristics as well as utilization of various carbon forms of PGPR strains isolated from the rhizosphere of wheat plants*

Isolate	Location[a]	Physiological and biochemical characteristics							Utilization of various carbon forms[f]							
		Colony Morphology[b]	Gram stain	Endos[c]	Catalase	Starch	VP[d]	Fermentation Test (glucose)[e]	G	S	Ml	F	Mn	X	T	R
WS01	FY	RE MW	–	ND	+	–	–	Acid	+	+	+	+	+	+	+	+
WS02	FY	RE MW	–	ND	+	+	–	–	+	+	+	+	+	+	+	+
WS03	FY	RE MW	–	ND	+	+	–	–	+	+	+	+	+	+	+	+
WS04	FY	IR MW	–	ND	+	+	–	–	–	–	–	+	+	–	–	+
WS05	HF	RE YW	–	ND	+	–	+	Acid	+	+	+	+	+	+	+	+
WS06	HF	RE SY	–	ND	+	+	–	–	+	+	+	+	+	+	+	+
WS07	FY	IR WH	–	ND	+	+	+	Acid	+	+	–	–	–	–	+	+
WS08	HF	RE MW	–	ND	+	+	–	–	+	+	–	+	+	+	+	+
WS09	HF	IR WH	–	ND	+	+	–	–	+	+	+	+	+	+	+	+
WS10	HF	RE MW	–	ND	+	+	–	–	+	+	+	+	+	+	+	+
WS11	HF	RE SY	–	ND	+	–	–	–	+	+	+	+	+	+	+	+
WS12	FY	IR MW	–	ND	+	+	+	Acid	+	–	+	–	+	+	+	+
WS13	FY	IR WH	+	+	+	+	–	–	–	+	–	–	+	–	+	+
WS14	FY	RE WM	+	+	+	+	–	–	+	+	+	+	+	+	+	+
WS15	HF	RE WM	–	ND	+	+	–	–	+	+	+	+	+	+	+	+
WS16	FY	IR WM	+	+	+	+	–	–	–	+	–	–	–	–	+	+
WS17	HF	RE VI	–	ND	+	+	–	Acid	+	+	+	+	+	+	+	+
WS18	FY	RE SY	–	ND	+	+	+	Acid	+	+	+	–	+	+	+	+
WS19	FY	RE MW	+	+	+	+	–	–	+	+	+	+	+	+	+	+
WS20	FY	RE MW	+	+	+	–	–	–	–	+	–	–	–	–	–	–
WS21	FY	IR SY	+	ND	+	+	–	–	+	+	+	+	+	+	–	+
WS22	FY	IR SY	+	ND	+	+	–	–	+	+	+	+	+	+	+	+
WS23	FY	IR MW	+	+	+	+	–	–	+	+	+	+	+	+	+	+
WS24	FY	IR MW	+	ND	+	+	–	Acid	+	+	+	+	+	+	+	+
WS25	FY	IR MW	+	+	+	+	–	–	+	+	+	+	+	+	+	+
WS26	FY	IR SY	+	+	+	+	–	–	–	+	–	–	–	–	+	+
WS27	FY	RE YW	+	ND	+	–	–	–	–	+	–	–	–	–	–	+
WS28	FY	IR MW	+	+	+	+	–	–	–	+	–	–	–	–	+	+
WS29	FY	IR SY	+	+	+	+	–	–	–	+	+	+	+	+	+	+
WS30	FY	IR WH	–	ND	+	+	–	–	–	+	+	+	+	+	+	+
WS31	FY	IR WH	+	+	+	+	–	–	–	+	+	+	+	+	+	+
WS32	FY	RE YW	–	ND	–	+	–	Acid	+	+	+	+	+	+	+	+

Note: * +, positive; –, negative
[a] FY, Fuyang; HF, Hefei
[b] RE, regular; IR, irregular; MW, milky white; WH, white; YW, yellowish white; SY, slight yellow; VI, vitelline.
[c] Endos, endspore; ND, not detected
[d] VP (Voges–Proskauer test).
[e] Utilization of glucose to produce acid.
[f] G, glucose; S, sucrose; Ml, maltose; F, fructose; Mn, mannose; X, xylose; T, trehalose; R, rhamnose

Table 2. Characterization of plant growth-promoting traits

Isolate	Plant growth promoting traits		
	IAA production[a] (mg/L)	Siderophore production[b]	Phosphate solubilization[c] (μg/ml)
WS01	-	+++++	18.91±0.43
WS02	10.27±0.53	-	25.94±0.36
WS03	12.03±0.56	+++++	18.96±0.27
WS04	12.45±0.55	-	28.87±0.32
WS05	81.43±1.52	++++	34.88±1.39
WS06	-	+++++	20.62±0.39
WS07	-	-	34.70±0.67
WS08	14.87±0.55	+++++	19.22±0.26
WS09	9.03±0.57	-	24.07±0.49
WS10	20.82±0.11	+++++	8.84±0.11
WS11	-	++++	-
WS12	103.35±1.08	+++	33.63±0.23
WS13	9.59±1.18	++	36.54±0.28
WS14	10.81±0.58	+++++	24.38±0.51
WS15	3.52±0.13	+++++	25.22±0.79
WS16	3.62±0.11	+++++	8.52±0.10
WS17	-	-	35.45±0.46
WS18	17.90±0.56	+++++	27.52±0.54
WS19	83.61±0.76	-	20.01±0.18
WS20	11.99±0.54	-	20.77±0.23
WS21	-	-	10.47±0.27
WS22	22.45±0.55	-	25.63±0.29
WS23	14.93±0.58	+++++	34.01±0.30
WS24	17.05±0.56	+++++	32.16±1.01
WS25	-	-	10.93±0.19
WS26	-	++	29.64±1.39
WS27	9.88±0.61	-	35.75±1.47
WS28	-	-	9.38 0±0.13
WS29	10.45±0.60	+	53.84±1.26
WS30	-	-	10.59±0.17
WS31	9.46±0.11	+	57.77±1.03
WS32	-	+++++	31.69±0.26

Note: [a] Production of IAA determined in liquid medium amended with L-tryptophan after 7 d of growth.
[b] +++++, very high; ++++, high; +++, moderate; ++/+, low; –, not detected.
[c] Amount of phosphorus solubilized into a modified Pikovskaya liquid medium.

manufacturer's instructions (http://www.sangon.com/, China). The following primers were used for the polymerase chain reaction (PCR) amplification of the 16S ribosomal DNA: fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and rP2 (5′-ACGGCTACCTTGTTACGACTT-3′) (Weisburg *et al.*, 1991). The PCR process was performed according to Park *et al.* (2005). The 16S rDNA nucleotide sequences were determined by Sangon Biotech (Shanghai) Co., Ltd.

The nucleotide sequences of the 16S rDNA were subjected to BLAST analysis with the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences with high similarity scores were downloaded from the RDP database (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp). A phylogenetic tree was constructed using MEGA 4.0 (Tamura *et al.*, 2007). All sequences were deposited in the GenBank sequence database, and the accession numbers are listed in Table 4.

Plant growth promotion

Wheat seeds (*Triticum aestivum* Wan 33) were surface sterilized according to a previous method (Yegorenkova *et al.*, 2001). The seeds were placed in sterile Petri dishes, properly watered, and germinated in darkness for 2 d at 25°C.

Germinated seeds were inoculated by immersion in appropriate bacterial suspensions (10^8 cells/ml) for 30 min at 28°C. Control seeds were soaked in distilled water, transferred to glass tubes (Φ3 cm×18 cm) containing 60 ml of semi-solid Hoagland medium (Hoagland and Arnon, 1950), and kept in a greenhouse at 25°C with a photoperiod of 16 h light and 8 h dark. The fresh and dry weights of shoots and rootswere calculated. The numbers of lateral roots were also recorded after 25 d.

Statistical analysis

The results of the measurements were subjected to ANOVA. Significance at the 5% level was tested by Fisher's least significant difference (LSD) using the Oringin Version 8.0 software.

Table 3. Comparison of plant growth-promoting (PGP) traits with previous reports

Total No.[a]	IAA			Siderophore		P-solubilization			IAA/Sid/P[e]		Plant	Reference
	No.[b]	Concen.[c] (mg/L)	Prop.[d] (%)	No.	Prop. (%)	No.	Concen. (mg/L)	Prop. (%)	No.	Prop. (%)		
32	**21**	**3.5 to 103.3**	**65.6**	**19**	**59.3**	**31**	**8.8-57.7**	**96.8**	**14**	**43.75**	wheat	This study
30	20	1.8 to 24.8	73	NR[f]	-	NR	-	-		-	wheat	Khalid *et al.* (2004)
4	2	100.5 to 112.8	50	NR	-	NR	-			-	wheat	Park *et al.* (2005)
7	7	1.9 to 42.1	100	1	14.3	1	188.7	14.3	1	14.3	wheat	Hafeez *et al.* (2006)
3[g]	1	6.75[h]	33.3	3	-	2	NR	-	1	33.3	wheat	Fischer *et al.* (2007)
9	6	to 27.5	66.7	NR	-	NR	-	-		-	wheat	Chopade *et al.* (2009)
8	8	5.5 to 31.0	100	NR	-	4	NR	50	0	-	wheat	Abbasi *et al.* (2011)
24	4	NR	16.7	6	25	5	NR	20.8	0	-	soybean	Cattelan *et al.* (1999)
80	27	9.5 to 80	33.8	39	48.7	46	NR	57.5	12	15	Veget[i]	Kumar *et al.* (2011)
155	14	NR	0.09	NR	-	NR	-	-	0	-	soil	Yuan *et al.* (2011)

[a] Total number reported in literature.
[b] Num, the number of isolates showing PGP properties.
[c] Concen, the amount of IAA, siderophores, and phosphorus produced by the isolates.
[d] Prop, isolates showing PGP properties in the total number.
[e] Isolate displaying the three PGP properties (IAA, siderophores, and phosphorus).
[f] NR, Not reported.
[g] There were 120 isolates reported, but only 3 were tested for PGP traits.
[h] Amount of IAA was 6.75 mg/ml.
[i] Vegetable crops.

Table 4. Identification of bacterial isolates based on 16S rDNA partial sequence analysis

Isolate	Organisms identified	Accession number	Closest type strain in RDP data base	16S rDNA identity (%)
WS03	*Arthrobacter* sp.	JN210899	*Arthrobacter defluvii*; AM409361	94.0
WS05	*Enterobacter* sp.	JN210900	*Enterobacter asburiae*; AB004744	95.6
WS06	*Pseudomonas* sp.	JN210901	*Pseudomonas mandelii*; AF058286	95.0
WS08	*Arthrobacter* sp.	JN688165	*Arthrobacter oryzae*; AB279889	95.2
WS10	*Arthrobacter* sp.	JN210902	*Arthrobacter oxydans*; X83408	96.3
WS11	*Variovorax* sp.	JN210903	*Variovorax paradoxus*; AJ420329	97.3
WS12	*Enterobacter* sp.	JN899570	*Enterobacter asburiae*; AB004744	94.9
WS13	*Bacillus megaterium*	JN688166	*Bacillus megaterium*; D16273	95.8
WS14	*Pseudomonas* sp.	JN688162	*Pseudomonas monteilii*; AF064458	97.3
WS15	*Arthrobacter* sp.	JN210904	*Arthrobacter ramosus*; X80742	95.1
WS17	*Dyadobacter fermentans*	JN210905	*Dyadobacter fermentans*; AF137029	96.1
WS19	*Bacillus megaterium*	JN688163	*Bacillus megaterium*; D16273	95.6
WS23	*Bacillus* sp.	JN210906	*Bacillus megaterium*; D16273	95.9
WS24	*Bacillus* sp.	JN210907	*Bacillus megaterium*; D16273	96.2
WS27	*Micrococcus endophyticus*	JN688164	*Micrococcus endophyticus*; AJ536198	94.6
WS29	*Bacillus* sp.	JN210908	*Bacillus circulans*; AY043084	80.1
WS31	*Bacillus pumilus*	JN210909	*Bacillus pumilus*; AY876289	98.8
WS32	*Pseudomonas* sp.	JN210910	*Pseudomonas costantinii*; AF374472	97.2

Note: Accession number, the accession number of the strains deposited in the GenBank (NCBI).

Results

Phenotypic characterization

Approximately 100 isolates were obtained from wheat rhizosphere by selective media, and 32 strains were positively reacted with WGA-FITC (Fig. 1). Out of the 32 strains, 17 were obtained from the modified Ashby medium, 11 from the potassium-releasing selective medium, and 4 strains from the modified Pikovskaya medium.

Most of the strains were Gram-negative, motile rods, and non-endospore forming. The colonies were milky white, white, yellowish white, etc. The oxidase test was positive for 31 isolates except WS15. The starch test was positive for 27 isolates, and the VP test was positive for 4 bacterial strains. Most isolates could utilize monosaccharides (such as glucose, maltose, etc.) and a disaccharide (sucrose) as carbon sources (Table 1).

Plant growth-promoting traits characterization

Out of 32 tested isolates, 21 (65.6%) strains were found to produce IAA, ranging from 3.5 mg/L to 103.3 mg/L. Three isolates producedmore than 80 mg/L IAA, and strain WS12 showed the highest IAA production (103.3 mg/L). Nineteen (59.3%) isolates produced siderophores, with 12 strains producing them at high amounts. Thirty-one (96.8%) strains exhibited the ability to solubilize phosphate. Strains WS29 and WS31 solubilized phosphate at 53.8 and 57.7 μg/ml, respectively (Table 2). All 32 isolates were compared with data from literatureon plant growth-promoting traits (Table 3). Fourteen isolates displayed three plant growth-promoting traits. Both the quantity and proportion of isolates possessing PGPR properties were higher than in previous reports.

Phylogenetic analysis

Based on the phylogenetic analysis of the 16S rDNA partial sequence, 6 strains were identified as *Bacillus* sp. Strain WS13, WS19, WS23, and WS24 were identified as *B. megaterium*. WS29 was *B. circulans*, and WS31 was *B. pumilus*. WS03 was *Arthrobacter defluvii*. WS08 and WS10 were *A. oxydans*. WS15 was *A. ramosus*. WS06 and WS14 were *Pseudomonas mandelii*. WS32 was *P. costantinii*. WS05 and WS12 were *Enterobacter* sp. WS11, WS17, and WS27 were *Variovorax* sp., *Dyadobacter fermentans*, and *Micrococcus endophyticus*, respectively (Table 4). Based on the neighbor-joining method, a total of 18 strains were clustered into seven groups (Fig. 2). The three major groups were *Arthrobacter*, *Bacillus*, and *Pseudomonas*.

Plant growth promotion

Two isolates displaying sufficient phosphorus production were chosen to determine their beneficial effects on wheat growth under greenhouse conditions. Wheat shoots grew better than controls when inoculated with the two isolates (data not shown). Strain WS29 greatly increased lateral root growth by 34.9%, and significantly increased root dry weight by 25.0% compared with the control treatment. However, the shoot dry weight was not evidently increased (Fig. 3A). Strain WS31 significantly increased the lateral roots by 27.6% and the root dry weight by 25.6% compared with the uninoculated control. Strain WS31 also slightly increased the shoot fresh weight compared with the control, but did not greatly increase the shoot dry weight (Fig. 3B).

Discussion

PGPR that stimulate plant growth via both direct and indirect mechanisms are widely considered as alternatives to common biofilitizers or biocontrol agents (Bano and Musarrat, 2003; Vessey, 2003). Numerous attempts have been made to isolate effective PGPR from various plants according to different criteria, such as auxin and siderophore production

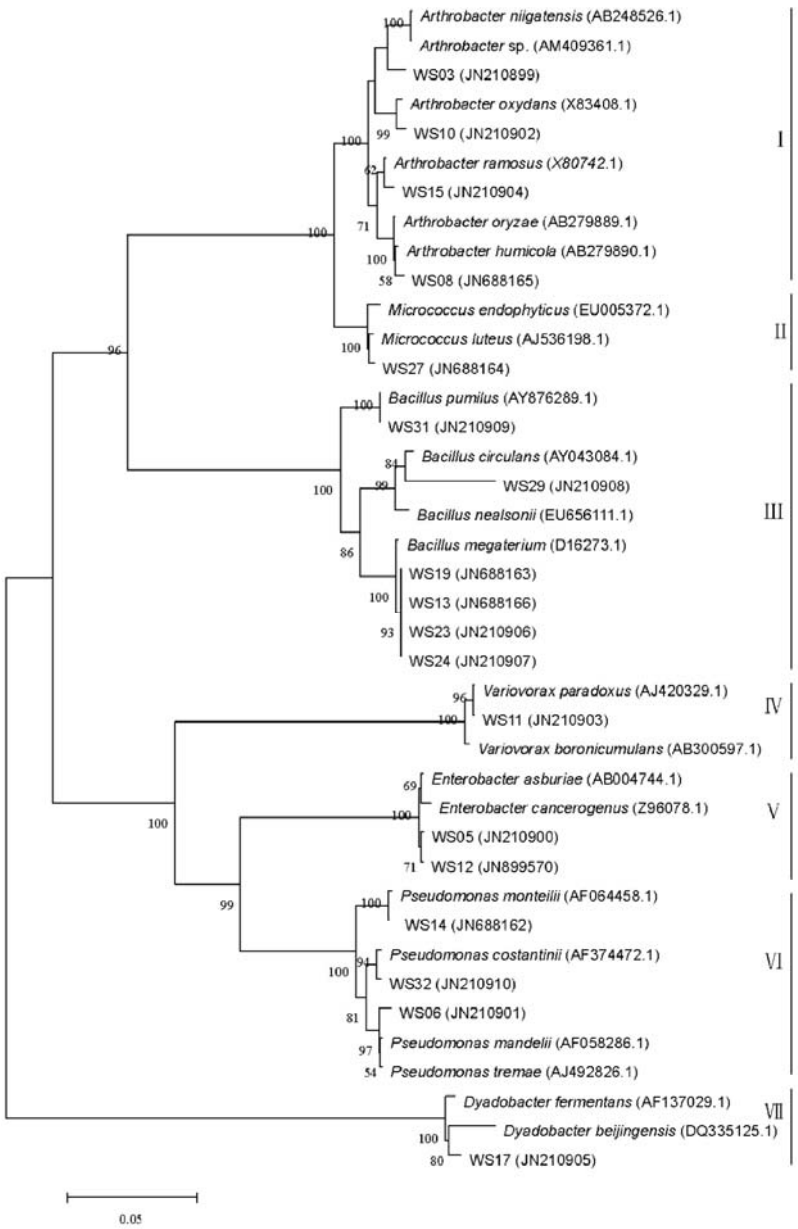

Fig. 2. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequence showing the position of isolated strains with the species of each genus downloaded from the RDP database. Bootstrap percentage values as obtained from 2,000 resamplings of the data set are given at the nodes of the tree. Only values greater than 50% are shown. Bar represents 0.05 substitutions per nucleotide position.

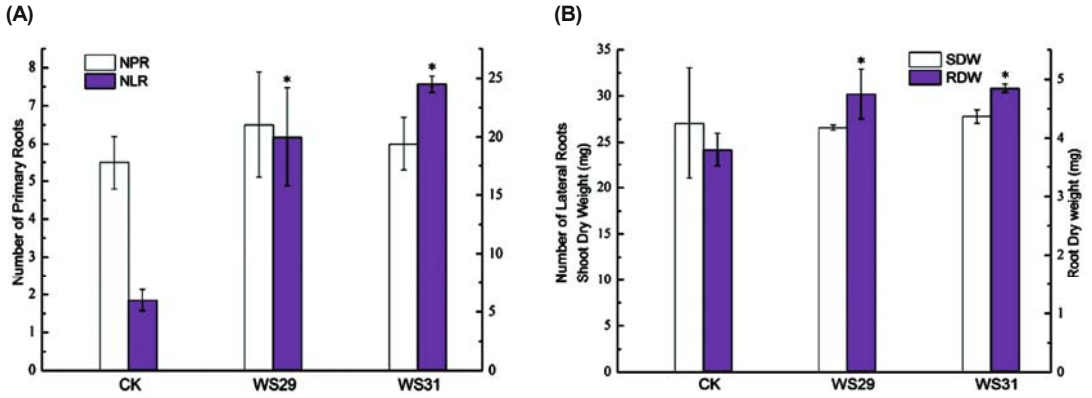

Fig. 3. Growth promotion effects of isolates on primary and lateral wheat roots, as well as on shoot and root dry weights: (A) lateral wheat roots and (B) root dry weight. NPR, number of primary roots; NLR, number of lateral roots; SDW, shoot dry weight; RDW, root dry weight. Significant differences tested according to Fisher's protected LSD at $*p=0.05$. Values are the means±standard deviations of three experiments.

or nitrogen-fixing activities. In fact, plants can also direct beneficial bacteria to adhere to their roots (Hartmann *et al*., 2009). However, most approaches to isolate PGPR from plant rhizosphere have not considered the possible relationship between plants and PGPR. Consequently, WGA was used in the present study as a tool for isolating highlyeffective PGPR strains from wheat rhizosphere according to the specific distinction between plant and rhizobacteria.

Thirty-two isolates were positively stained by WGA-FITC, suggesting that these bacteria possessed specific attachment sites for WGA. Considering wheat plants, the amount of isolates in the current study was almost the same as that reported by Khalid *et al*. (2004), but higher than in other reports (Park *et al*., 2005; Hafeez *et al*., 2006; Fischer *et al*., 2007; Chopade *et al*., 2009; Abbasi *et al*., 2011). This finding indicated that WGA-FITC can be a useful tool for isolating more PGPR strains for wheat. Compared with other crops, the amount of strains obtained in the presented study was higher than those reported by Cattelan *et al*. (1999), but less than those of others (Kumar *et al*., 2011; Yuan *et al*., 2011). However, bacteria are greatly diverse among different crops. This diversity can affect the quantity of bacteria isolated from different plants.

In the current study, 65.6% of the isolates produced IAA, 59.3% produced siderophores, and 96.8% solubilized tricalcium phosphate. Although the IAA concentrations produced by the isolates in the present study were not as high as those reported by Park *et al*. (2005) and Fischer *et al*. (2007), the quantities of the strains that produced IAA were larger. The soluble phosphate released was also lower than that reported by Hafeez *et al*. (2006). However, 43% of isolates displayed three types of PGPR traits. The proportion of isolates showing PGPR properties was higher than in previous reports. The total number of isolates in the current study was lower than in previous reports. Nonetheless, more effective strains were re-isolated from a large amount of isolates by WGA-FITC.

Most of the strains displaying plant growth-promoting traits were identified based on a 16S rDNA partial sequence. Out of 18 sequenced isolates, 6, 4, 3, and 2 isolates belong to the cluster of *Bacillus*, *Arthrobacter*, *Pseudomonas*, and *Enterobacter* spp., respectively. There was one isolate for each of the genera *Variovorax* sp., *Dyadobacter* sp., and *Micrococcus* sp. The number of isolates belonging to the genus *Bacillus* was higher than those from other groups, similar with the report that *Bacillus* spp. are dominant in root-adhering soil (Laguerre *et al*., 1994). A total of seven genera were identified in the present study, suggesting that these bacterial strains isolated by WGA-FITC also presented diversity.

Due to the low solubility of phosphorus in soil, two phosphorus-dissolving isolates were chosen to evaluate their plant growth promotion effects under greenhouse conditions. The inoculation of isolate WS31 significantly increased the root dry weight. Both WS29 and WS31 greatly increased the number of lateral roots, but failed to increase wheat shoot fresh and dry weights. This finding indicated that rhizobacteria can stimulate early root growth in the wheat seedling stage. Based on 16S rRNA gene sequence comparisons and phylogenetic positions, both isolates WS29 and WS31 were determined to belong to the genus *Bacillus*. In this genus, there are reported strains possessing phosphate-dissolving abilities (Hafeez *et al*., 2006; Behbahani, 2010; Dhandapani, 2011).

Conclusions

In the present study, a total of 32 isolates were positively stained by WGA-FITC. The proportion of the isolates displaying PGPR traits was higher than those in previous reports. The efficiency of the isolation of effective PGPR strains was greatly increased by WGA. This result indicates that WGA can serve as an effective tool for isolating more efficient PGPR strains from wheat plant roots. These findings can facilitate further research on highly effective biofertilizers or biocontrol agents.

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