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Diazotrophic *Paenibacillus beijingensis* BJ-18 Provides Nitrogen for Plant and Promotes Plant Growth, Nitrogen Uptake and Metabolism

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Diazotrophic bacteria can reduce N_2 into plant-available ammonium (NH_4^+), promoting plant growth and reducing nitrogen (N) fertilizer requirements. However, there are few systematic studies on the effects of diazotrophic bacteria on biological N_2 fixation (BNF) contribution rate and host plant N uptake and metabolism. In this study, the interactions of the diazotrophic *Paenibacillus beijingensis* BJ-18 with wheat, maize, and cucumber were investigated when it was inoculated to these plant seedlings grown in both low N and high N soils, with un-inoculated plants as controls. This study showed that GFP-tagged *P. beijingensis* BJ-18 colonized inside and outside seedlings, forming rhizospheric and endophytic colonies in roots, stems, and leaves. The numbers of this bacterium in the inoculated plants depended on soil N levels. Under low N, inoculation significantly increased shoot dry weight (wheat 86.1%, maize 46.6%, and cucumber 103.6%) and root dry weight (wheat 46.0%, maize 47.5%, and cucumber 20.3%). The ^{15}N-isotope-enrichment experiment indicated that plant seedlings derived 12.9–36.4% N from BNF. The transcript levels of *nifH* in the inoculated plants were 0.75–1.61 folds higher in low N soil than those in high N soil. Inoculation enhanced NH_4^+ and nitrate (NO_3^-) uptake from soil especially under low N. The total N in the inoculated plants were increased by 49.1–92.3% under low N and by 13–15.5% under high N. Inoculation enhanced activities of glutamine synthetase (GS) and nitrate reductase (NR) in plants, especially under low N. The expression levels of N uptake and N metabolism genes: *AMT* (ammonium transporter), *NRT* (nitrate transporter), *NiR* (nitrite reductase), *NR*, *GS* and *GOGAT* (glutamate synthase) in the inoculated plants grown under low N were up-regulated 1.5–91.9 folds, but they were not obviously changed under high N. Taken together, *P. beijingensis* BJ-18 was an effective, endophytic and diazotrophic bacterium. This bacterium contributed to plants with fixed N_2, promoted plant growth and N uptake, and enhanced gene expression and enzyme activities involved in N uptake and assimilation in plants. However, these positive effects on plants were regulated by soil N status. This study might provide insight into the interactions of plants with beneficial associative and endophytic diazotrophic bacteria.

Keywords: diazotroph, ^{15}N isotope enrichment, biological N_2 fixation, colonization, GFP, wheat, maize, cucumber

INTRODUCTION

Biological nitrogen (N) fixation (BNF) is the major natural process through which atmospheric N_2 is reduced to bioavailable NH_4^+, providing a large amount of natural N into cultivated agricultural systems (Galloway et al., 2008). In addition to symbiotic N_2-fixing *Rhizobia* associated with legumes, the non-symbiotic diazotrophic bacteria are also important contributors to the N nutrition of non-legumes (Gupta et al., 2006). It is estimated that the microbial N accounts for roughly 30–50% of the total N in crop fields (Liu et al., 2017). The non-symbiotic diazotrophic bacteria are highly diverse and associated with plants in different ways. Some bacteria live in the rhizosphere and are designated rhizobacteria (Kloepper and Beauchamp, 1992). *Herbaspirilla seropedicae* strain Z67 mainly colonize on the riceroot surface and are usually called associative diazotrophic bacteria (Monteiro et al., 2012). *Paenibacillus polymyxa* WLY78 live inside the plant without causing damage and are classified as endophytic diazotrophic bacteria (Hao and Chen, 2017). Endophytic diazotrophic bacteria may have an advantage over associative diazotrophic bacteria and rhizobacteria, since they live within plant tissues where better niches are established for N_2 fixation and assimilation of fixed N_2 by the plant (Reinhold-Hurek and Hurek, 1998, 2011).

The well-known associative and endophytic diazotrophic bacteria include *Azospirillum* (Boddey et al., 1986), *Azoarcus* (Hurek et al., 2002), *Burkholderia* (Baldani et al., 2000), *Enterobacter* (Magnani et al., 2010), *Gluconacetobacter* (James et al., 2001), *Herbaspirillum* (Boddey et al., 1995). BNF quantification experiments show that associative and endophytic bacteria can fix N_2 in plant tissues with higher efficiency (Carvalho et al., 2014). *G. diazotrophicus* inoculation enhanced sugarcane yield by providing 50–80% N from BNF (Boddey et al., 1995). It is estimated that an 18–28% of plant N derives from BNF of endophytic *Enterobacter* sp. strain (Mirza et al., 2001). Diazotrophic bacteria present in the mucilage of aerial roots contribute 29–82% of the N nutrition of Sierra Mixe maize (Van Deynze et al., 2018).

Although the positive effects of diazotrophic bacteria on plants are observed, little is known about plant response to inoculation with diazotrophic bacteria. Plant N metabolism is a complex process requiring some key enzymes. The plant genes *NR* (nitrate reductase), *NiR* (nitrite reductase), *GS* (glutamine synthetase) and *GOGAT* (glutamate synthase) play very important roles in N metabolism (Bloom et al., 1992; Lea and Miflin, 2003). The plant genes *AMT* (ammonium transporter) (Bloom et al., 1992) and *NRT* (nitrate transporter) (Sugiura et al., 2007) are involved in N uptake. It is shown that some endophytic fungi affect expression of N metabolism of plants (Yang et al., 2014).

Paenibacillus beijingensis BJ-18, isolated from wheat rhizosphere, was a N_2-fixer (Wang et al., 2013). Inoculation with *P. beijingensis* BJ-18 promoted the growth of tomato seedlings (Xie et al., 2016) and increased wheat yield by 26.9% in field experiment (Shi et al., 2016), suggesting that this bacterium promotes plant growth. It was generally recognized that plant growth-promoting bacteria (PGPB) promoted plant growth by direct mechanisms (e.g., N fixation, phosphate solubilization, sequestering iron) and indirect mechanisms [e.g., indole-3-acetic acid (IAA), cytokinins, gibberellins] (Glick, 2012). However, the mechanisms utilized by *P. beijingensis* BJ-18 to promote plant growth were not clear. In this study, we investigated the colonization pattern and contributions of N_2 fixation by *P. beijingensis* BJ-18 to plants, and the plant responses (N uptake and metabolism processes) to the infection.

MATERIALS AND METHODS

Bacteria Strains and Growth Conditions

Paenibacillus beijingensis BJ-18 (accession number: JN873136), isolated from wheat rhizosphere, is a novel species with N_2-fixing ability [1043 ± 12.9 nmol C_2H_4 (mg protein h)$^{-1}$] (Wang et al., 2013). This bacterium has multiple antagonistic activities against plant pathogens and produces IAA (24.95 μg mL^{-1}) (Xie et al., 2016). The bacterial suspension of *P. beijingensis* BJ-18 used in inoculation was prepared as follows. The *P. beijingensis* 1–18 cells were cultured overnight in Luria-Bertani (LB) broth at 30°C and 180 rpm, and then cells in the logarithmic growth phase were harvested by centrifugation and finally the pellet was suspended with sterile normal saline (0.89% w/v NaCl in double distilled water) to the final concentration at 10^8 cells mL^{-1}.

Colonization of GFP-Tagged *P. beijingensis* BJ-18 in Wheat, Maize, and Cucumber Tissues

The recombinant plasmid pGFP300 carrying *gfp* gene (Hao and Chen, 2017) was introduced into *P. beijingensis* BJ-18 by electrotransformation (Zhang et al., 2013), yielding GFP-tagged *P. beijingensis* BJ-18. And the physiological ability of GFP-tagged *P. beijingensis* BJ-18 was not changed, compared with wild-type *P. beijingensis* BJ-18 (data not published). The GFP-tagged *P. beijingensis* BJ-18 suspension was obtained as described above. Plump seeds of wheat "Jimai 22" (Shandong Runfeng Seed Industry Co., Ltd), maize "Zhengdan 958" (Henan Shangke Seed Co., Ltd.) and cucumber "Zhongnong 8" (Beijing Shengfeng Garden Agricultural Technology Co., Ltd) were surface-disinfected with 10% sodium hypochlorite for 10 min, followed by rinsing with sterilized water three times, and grown on the sterile petri dishes containing moist filter papers in darkness at room temperature (25°C) for 3–5 days, respectively. These plant seedlings had two treatments: inoculation with GFP-tagged *P. beijingensis* BJ-18 (E+) and mock inoculation (E-). For inoculation, the plant seedlings were soaked in bacterial suspension (10^8 cells mL^{-1}) of GFP-tagged *P. beijingensis* BJ-18 for 30 min to facilitate colonization. For mock inoculation, the plant seedlings were soaked in sterilized deionized water for 30 min. The germinated wheat, maize and cucumber seeds were, respectively, sown in sterile flask (3 seeds per flask, 6 cm in diameter and 10 cm in height) containing 100 mL 1/2 × Murashige and Skoog semisolid agar medium (Prod

No: M519, PhytoTechnology Laboratories, Shawnee Mission, United States) (Murashige and Skoog, 1962). Then these seedlings were grown in the light growth chamber (27°C, 70% humidity and 16 h day/8 h night, with light at 250 μmol m^{-2} s^{-1}). The GFP-tagged *P. beijingensis* BJ-18 in plant tissues was observed at 2 weeks after inoculation by confocal laser scanning microscopy (CLSM, Olympus FluoView^{TM} FV1000 confocal microscope, Olympus Corporation, Tokyo, Japan). These images were collected using FV10-ASW software (03.01.02.02, Olympus Europa Holding GmbH, Hamburg Germany) and processed in Adobe Photoshop CC 2015 and Adobe Illustrator CS6 (Adobe, San Jose, CA, United States).

Plant Culture and Collection

Seedling growth assays were performed in plastic pots (diameter of 35 cm; height of 25 cm) filled with 5 kg non-sterile soil which was top soil (0–20 cm depth) taken from the Shangzhuang Experimental Station of China Agricultural University, Beijing, China (40°08′12.15″ N, 116°10′44.83″ E, 50.21 m above sea level). The soil was low N-content sandy loam (N_{min}, 7.8 mg kg^{-1}; Olsen-P, 7.3 mg kg^{-1}; NH_4OAc-K, 115.8 mg kg^{-1}; O.M., 7.2 g kg^{-1}; pH 7.7; E.C., 0.4 dS m^{-1}). The soil was air-dried, crushed, and screened by a 2 mm sieve to remove debris and reduce heterogeneity for cultivating plants: wheat, maize and cucumber. Before planting, P (Na_2HPO_4) and K (KCl) fertilizer were applied to soil as base fertilizers at amounts of 50 mg P and 17 mg K per kg soil, respectively, based on the recommendation by Ke et al. (2018). The microelements were not applied to soil during plant growth.

The experimental design was randomized with factorial arrangement (a 2 × 2 factorial design) in three replications with bacterial factor at two levels and N factor at two levels. Three different plants (wheat, maize, and cucumber) were chosen to obtain an objective conclusion. Therefore, the experiments had twelve treatments.

The seeds (wheat, maize, and cucumber) were germinated as described above. After germination, vigorous and homogenous seedlings were chosen for transplanting into plastic pots. These plant seedlings had two treatments: inoculation with *P. beijingensis* BJ-18 (E+) and mock inoculation (E-). For inoculation, the plant seedlings were soaked in bacterial suspension (10^8 cells mL^{-1}) of *P. beijingensis* BJ-18 for 30 min to facilitate colonization. For mock inoculation, the plant seedlings were soaked in sterilized deionized water. Then the inoculated seedlings and un-inoculated seedlings were, respectively, transplanted into pots (cucumber: 4 seedlings per pot; maize: 2 seedlings per pot; wheat: 4 hills per pot and 10 seedlings per hill). On day 7, 80 ml of the bacterial suspension was applied to pot containing inoculated seedlings and 80 mL of sterile water was applied to pot containing non-inoculated seedlings. Each of inoculation and mock inoculation treatments had three replicates.

There were two levels of N treatments: high N level (250 mg N kg^{-1} soil) and low N level (83 mg N kg^{-1} soil). The ^{15}N-labeled $(NH_4)_2SO_4$ (10.16% ^{15}N atom, Shanghai Research Institute of Chemical Industry, China) was applied to soils in all pots. The N fertilizer was added in three separate applications: the first application was done before planting as base fertilizer (approximately 33.3% of total N), and successive two applications (approximately 33.3% of total N per time) were made on day 7 and 14 after transplanting, respectively. Pots were placed in the greenhouse under optimum conditions (15 h light/9 h dark cycle, 25–30/15–20°C day/night temperature and 40% day/60% humidity). The seedlings were regularly watered (tap water) to 40% relative soil moisture by weighing method every 5 days.

The samples of wheat, maize, and cucumber were harvested from each treatment on day 35 after transplanting, respectively.

Firstly, the whole seedling was uprooted, and then shoot and root samples were separated and washed with deionized water to remove the adhering soil. Shoot and root samples were oven-died at 105°C for 30 min to inactivate the enzyme, respectively, followed by 65°C until constant weight for dry weight analysis. Then, the oven-dried samples were grinded, screened by a 1 mm sieve, and stored in zip-lock bag for plant N content and ^{15}N enrichment determination. Afterward, the remaining samples were immediately frozen in liquid N and then maintained at −80°C until further analysis.

Bacterial Cell Concentration Within Plant Tissues

The cell densities of diazotrophic *P. beijingensis* BJ-18 within the inoculated plant tissues were estimated by qPCR according to the method described by Rasmussen et al. (2007). Primers for qPCR of the *nifB* from *P. beijingensis* BJ-18 included *nifB* F (5′-GAAGGTGAGAGTGAGGATGG-3′) and *nifB* R (5′-TTGCTTCAGGCTCATCTCC-3′). qPCR was performed with plant genomic DNA as template which was extracted form plant seedlings using DNA Kit [TianGen Biotech (Beijing) Co., Ltd.]. The 129 bp PCR product was ligated to the PMD 19-T vector (Takara, Otsu, Japan) and then introduced into *Escherichia coli* JM109. The introduced *E. coli* JM109 was grown in liquid LB medium, and the recombinant plasmids carrying *nifB* fragment were extracted and purified using TIANprep Mini Plasmid Kit [TianGen Biotech (Beijing) Co., Ltd.]. A standard curve was generated for each run 10-fold dilution series from 2×10^1 to 2×10^7 copies. The plant genomic DNA isolated from each of the different treatments was mixed with SYBR® Premix Ex TaqTM (Takara, Kyoto, Japan), primer pairs and ddH_2O in a total volume of 20 uL for qPCR on a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, United States). Ct values were measured to quantify initial amounts of target DNA.

Quantification of Biological N_2 Fixation Contribution

In this study, BNF contribution of *P. beijingensis* BJ-18 to plants was quantified by the method of ^{15}N isotope dilution technique. N content and ^{15}N enrichment in plant tissues were determined by DELTA V Advantage isotope ratio mass spectrometer (Thermo Fisher Scientific, Inc., United States). The plants without *P. beijingensis* BJ-18 inoculation were used as references to calculate the BNF contribution. The BNF

contribution was calculated according to formula 1 described by Boddey and Knowles (1987):

$$\%\text{Ndfa} = \left(1 - \frac{\%^{15}\text{Na.e.}_{\text{I}}}{\%^{15}\text{Na.e.}_{\text{UI}}}\right) \times 100 \quad (1)$$

Where, %Ndfa is the percentage of N derived from air and percent ^{15}Na.e. ($\%^{15}$N atom excess) is the enrichment of the inoculated (I) and un-inoculated (UI) plants, respectively.

Determination of the Concentration of Free NH_4^+, NO_3^-, and Activities of GS and NR

In order to determine the concentrations of the free ammonium (NH_4^+) and nitrate (NO_3^-), fresh plant tissues were ground with a mortar on ice in extraction buffer. The buffer consisted of 10 mM imidazole, 50 mM Tris-HCl (pH 8.0), and 0.5% (w/v) β-mercaptoethanol. After grinding, the samples were centrifuged at 12,000 × *g* for 20 min at 4°C and the supernatant was collected for free NH_4^+ and NO_3^- determination (Oliveira et al., 2002; Yang et al., 2014). Free NH_4^+ concentration [$\mu g\ g^{-1}$ fresh weight (FW)] in the supernatant was assayed using the Berthelot color reaction method (Gordon et al., 1978), and free NO_3^- concentration ($\mu g\ g^{-1}$ FW) in the supernatant was determined using the Griess method (Eckhardt et al., 1999).

For analysis of GS activity, fresh plant tissues were ground with a mortar in pre-cold extraction buffer (containing10 mM $MgSO_4$, 2 mM dithiothreitol, 70 mM 3 (n-morpholino) propane-sulfonic acid (pH 6.8), 5 mM glutamate, 10% (v/v) ethanediol, 0.1% (v/v) TritonX-100). The extracts were centrifuged at 12,000 × *g* for 30 min at 4°C and the supernatant was collected for plant GS activity determination using NH_2OH as a substrate, and the amount of γ-glutamyl hydroxamate (GHA, ug^{-1} FW min^{-1}) released was determined spectrophotometrically at 540 nm according to the method of Yang et al. (2014).

To measure NR activity, fresh plant tissues were ground on ice in extraction buffer consisting of 25 mM phosphate buffer (pH 7.5, a mixture of K_2HPO_4 and KH_2PO_4), 5 mM cysteine and 5 mM EDTA-Na_2. The mixture was centrifuged at 4,000 × *g* and 4°C for 10 min and the supernatant was collected. NR activity was measured spectrophotometrically at 540 nm according to Yu and Zhang (2012). NR activity was expressed as $\mu g\ NO_2^-\ g^{-1}$ FW h^{-1}.

Quantitative Real-Time PCR Analysis of Plant Genes and *nifH* in Plant Roots and Shoots

Total RNA was extracted from plant tissues using RNAiso Plus reagent (RaKaRa, Kyoto, Japan). Then, RNA was digested with DNase I and reversely transcribed into cDNA using PrimeScript^{TM} RT reagent kit (RaKaRa, Kyoto, Japan). Gene expression levels were determined by quantitative real-time PCR (qRT-PCR) analysis. The specific primers for qRT-PCR were shown in **Table 1**. The plant housekeeping gene *actin* was used as plant internal control, and the bacterial 16S rRNA was used as bacterium internal control. The relative expression of the target genes were calculated according to the standard comparative C(t) method (Livak and Schmittgen, 2001). Each treatment had three biological replicates, with three technical replicates for each biological replicate.

Statistical Analysis

Statistical tests were performed using SPSS software version 20 (SPSS Inc., Chicago, IL, United States). Two-way analysis of variance (ANOVA) was employed to check the significant differences between treatments. Means of different treatments were compared using the least significant difference (LSD) at 0.05 or 0.01 level of probability. Graphs were prepared using SigmaPlot software version 12.5 (Systat Software, Inc., CA, United States).

RESULTS

Colonization of GFP-Tagged *P. beijingensis* BJ-18 in Wheat, Maize, and Cucumber Tissues

The CLSM observation showed that the GFP-tagged *P. beijingensis* BJ-18 cells emitted bright green fluorescence (**Figure 1a**). The GFP-tagged *P. beijingensis* cells were found to colonize on the surface of the primary roots and the root hair zone of wheat (**Figures 1b,c**). The bacterial cells were found to be distributed within cortex of wheat primary roots (**Figure 1d**) and colonized in the wheat vascular bundle (**Figure 1e**). Moreover, bacterial cells were observed in the vascular bundle of wheat stem (**Figures 1f,g**) and in wheat leaf vein (**Figure 1h**). In the maize seedlings, the *P. beijingensis* cells were found to colonize the surface of the primary roots (**Figure 2a**), and on the junction of the primary and lateral roots (**Figure 2b**). The bacteria cells were found within the intercellular spaces and xylem vessels of maize roots and stems (**Figures 2c–e**) and leaves (**Figure 2f**). The colonization pattern in cucumber was similar to those obtained in wheat and maize seedlings. The bacterial cells were found to colonize the surface of the primary roots and the root hair zone (**Figure 3a**). The *P. beijingensis* cells were in cortex (**Figure 3b**) and vascular bundle (**Figure 3c**) of cucumber roots. As shown in **Figures 3d,e**, bacterial cells invaded the xylem vessels of cucumber stem. Moreover, the bacterial cells were found within the cucumber leaves (**Figure 3f**).

Taken together, *P. beijingensis* cells colonized on the surface of roots and within roots, stems and leaves of wheat, maize, and cucumber, indicating that the colonization patterns in the three plants were similar.

Concentration of Diazotrophic *P. beijingensis* BJ-18 in Wheat, Maize, and Cucumber Tissues

The concentration of *P. beijingensis* BJ-18 in the inoculated plants at 35 day after inoculation was assessed using qPCR and was expressed as the number of copies of the specific *nifB* genes per total (plant seedlings + bacteria) genomic DNA. As shown in **Figure 4**, copy numbers of *nifB* gene in the

TABLE 1 | Primers sequence and accession number in NCBI.

Primer	Primer sequence 5′-3′	Size (bp)	NCBI Accession No.	References
CsAMT1	TTCTCTATCAGTGGGCTTTCG AGAACCAATGGGACACAACC	141	AY642427	This study
CsAMT3	AAGGTAGACGACACAATGG CGTAGAAGATGTTGTTGAGG	109	XM_004138819	This study
CsNRT 1.3	CACAAGCCTTCAGAGAATTGG TCAACCAGAAAGCACTTATACG	131	JX206800	This study
CsNR1	GCACAACTCAGACCAATCC GATGAGAATGCTGTCCATACC	103	HM755943	This study
CsNR2	TGTGCGTGTATTCAGATTCG GTGCTAGAGGGCGTATAGG	132	HM755944	This study
CsNiR	AGGATTGGTAGCTTGCACTGG ACTGTGACTCGCCGTTGC	105	EF397679	This study
CsGS1	ATGAGGGAAGAAGGAGGTTACG AGAGAAGGTGTGGATGTCAGC	147	JQ277263	This study
CsGOGAT	GGCTGCTCAAGGAAAGGAACC TGCTGGATTTGTCACCTGTGC	127	DQ641082	This study
TaAMT1.1	ACAGCTTCTTCCTCTTCC CCGAGTAGATGAGGTAGG	105	AY525637	This study
TaNRT1.1	ATGCCAGGTTGTCATTGC CCGAGTCCAGTTGTATGC	135	AY587265	This study
TaNRT2.1	TGGACTCCGAGCACAAGG GACGAAGCAGGTGAAGAAGG	104	AF288688	This study
TaNRT2.3	TGGTCAGAGGAGGAGAAGG GTGGCGAGGATAACATTGC	101	AY053452	This study
TaNR	ATACACCATGAAAGGATACG TACTTGTTCGGCTTCTCC	126	KY244026	This study
TaNiR	CTACACCAACCTCCTCTCC GCCAGGTCGTTGATATGC	138	FJ527909	This study
TaGS1	CCTTGTCATGTGCGATTGC GTGTACTCCTGCTCGATACC	135	DQ124211	This study
Ta GOGAT	AAACCAAGGGACCTCAGTATTC AATGACCACCACCTTCTTACC	150	DW986179	This study
ZmAMT1;1a	CATCGTCGGAAGGTGTGG TTGGATGATGAGCAGTGACC	109	GRMZM2G175140	This study
ZmAMT1;1b	CTACGACTTCTTCCTATACC CGGAGTAGATGAGGTAGG	111	GRMZM2G118950	This study
ZmNrt2.1	TGGACTCAGAGCACAAGG CGAAGCAGGTGAAGAAGG	102	AY129953	This study
ZmNR	GCCAGCATTGAAGGGAAG GCTCGTTCTTGAAGTAGACC	106	M77792	This study
ZmGS1-3	CTTGTGATGTGCGATTGC CTCCTGCTCAATACCATACC	129	X65928	This study
ZmGS1-4	AGGCATCAACATCAGTGG AGAATGTAGCGAGCAACC	117	X65929	This study
Zm GOGAT	*CGCTCTTCTGGCAACTGG* *CACCTTCCTGTAGTCTGATTCG*	133	M59190	This study
CsACTIN	AGAGATGGCTGGAATAGAAC CTGGTGATGGTGTGAGTC	333	DQ641117	Wei et al. (2015)
ZmACTIN	CATGGAGAACTGGCATCACACCTT CTGCGTCATTTTCTCTCTGTTGGC	118	J01238.1	Galli et al. (2013)
TaACTIN	GTCGGTGAAGGGGACTTACA TTCATACAGCAGGCAAGCAC	187	AB181991.1	Moloudi et al. (2013)

(Continued)

TABLE 1 | Continued

Primer	Primer sequence 5′-3′	Size (bp)	NCBI Accession No.	References
nifB	GAAGGTGAGAGTGAGGATGG TTGCTTCAGGCTCATCTCC	88	MH202771	This study
nifH	GCAACAGTCGGAATACGG TTGGGTCACGGTCATACG	136	MH555146	This study

inoculated plant tissues under low N level were much higher than under high N level. Wheat had the highest copy numbers of *P. beijingensis nifB* gene, followed by cucumber and then maize under both low N and high N levels. Copy numbers of *nifB* gene in roots of the three plants were much higher (62.5–185.3%) than in shoots under both low N and high N levels. These data suggested that the concentrations of the bacterial cells in plant tissues were related to soil N levels, plant species and plant tissues.

Plant Growth Promotion

To assess the impacts of *P. beijingensis* inoculation on plant growth, the biomass (dry weight) was analyzed in inoculated and un-inoculated plants under low N and high N levels. Dry weights of wheat shoots and in roots under low N were increased by 86.1 and 46.0%, respectively. Dry weights of maize shoots and in roots under low N were increased by 46.6 and 47.5%, respectively. Dry weights of cucumber shoots and in roots under low N were increased by 103.6 and 20.3%, respectively. The data suggested that *P. beijingensis* BJ-18 could efficiently promote the growth of the three plants (**Figure 5**). The increased shoot and root biomass by *P. beijingensis* BJ-18 inoculation under low N condition were significantly higher than those under high N condition, consistent with the concentration of *P. beijingensis* BJ-18 in these plant tissues under different N conditions.

Quantification of BNF in the *P. beijingensis*-Inoculated Wheat, Maize, and Cucumber

To estimate the contribution of BNF, ^{15}N isotope enrichment analysis was conducted to analyze inoculated seedling of wheat, maize and cucumber grown in soil contain ^{15}N-labeled $(NH_4)_2SO_4$ as N fertilizer in greenhouse conditions, in comparison with un-inoculated plants. As shown in **Table 2**, the roots and shoots of seedlings inoculated with *P. beijingensis* BJ-18 had significantly lower $\delta^{15}N$ value than un-inoculated seedlings under both low and high N conditions, suggesting that these plants derived a portion of N from atmospheric N_2. The plant N derived from the atmosphere (%Ndfa) ranged from 18 to 36.4% under low N level and from 12.9 to 30.5% under high N level. Among the three plants, wheat showed maximum %Ndfa (30.5 and 36.4%) under both high N and low N levels, followed by cucumber (25.4 and 27.8%) and then maize (12.9 and 20.9%). These data suggest that the contribution of BNF was closely related to soil N levels and to plant species. These results were in agreement with the concentration of *P. beijingensis* BJ-18 within plants and the plant biomass under different N levels.

Transcript Levels of *nifH* Gene

To investigate whether *nif* genes coding nitrogenase of *P. beijingensis* BJ-18 were expressed within plant tissues, the transcript levels of *nifH* gene, one of *nif* genes coding Fe protein of nitrogenase, were quantified. As shown in **Figure 6**, transcripts of *nifH* under low N were up-regulated in wheat shoots and roots by 1.09 and 1.61 folds, respectively, in maize shoots and roots by 0.77 and 1.0 folds, respectively, and in cucumber shoots and roots by 0.75 and 1.61 folds, respectively, compared to those under high N. The results suggested that soil N status affected transcript level of *P. beijingensis nifH*. The data were consistent with BNF rate and the concentration of *P. beijingensis* BJ-18 in plants grown in soils containing different N levels.

Promotion of N Uptake and Total N Content in Plants by Inoculation With *P. beijingensis* BJ-18

Compared to un-inoculated shoots and roots under low N level, a significant increase of free NH_4^+ concentration was observed in shoots of wheat (26.6%), maize (21.9%) and cucumber (22.2%) and in roots of wheat (24.9%), maize (52.7%) and cucumber (32.2%) (**Figure 7A**). In contrast, inoculation did not significantly enhance free NH_4^+ concentration in plant tissues under high N (**Figure 7B**).

Similarly, *P. beijingensis* inoculation significantly increased the free NO_3^- concentration in shoots of wheat (26.3%), maize (23.4%) and cucumber (43.7%) and in roots of wheat (60.7%), maize (62.6%) and cucumber (67.4%) under low N (**Figure 7C**). In contrast, inoculation did not significantly enhance free NO_3^- concentration in plant tissues under high N (**Figure 7D**).

Compared to un-inoculated shoots under low N level, a significant increase of total N content was observed in inoculated shoots of wheat (76.3%), maize (49.1%) and cucumber (88.8%) (**Figure 7E**). Similarly, inoculation greatly enhanced total N content in inoculated roots of wheat (61.6%), maize (68.9%) and cucumber (92.3%) (**Figure 7F**). In contrast, the increased levels of total N content in root and shoots were lower under high N (**Figures 7E,F**). The data were consistent with the change of the concentrations of the free NH_4^+, and free NO_3^- under low N and high N.

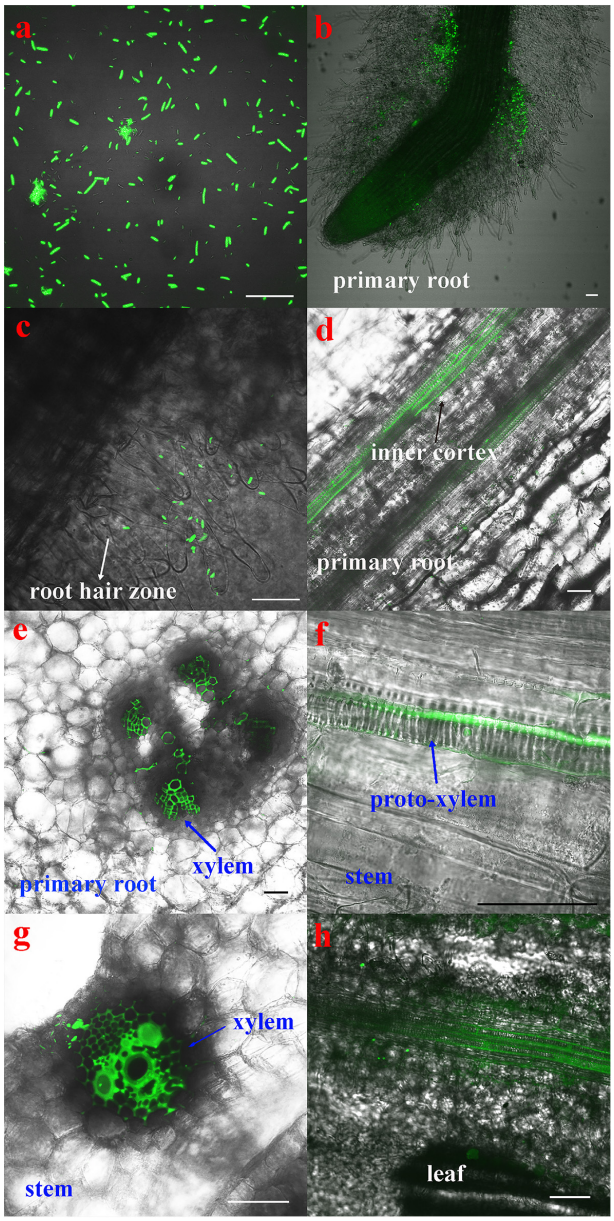

FIGURE 1 | Colonization of the GFP-tagged *P. beijingensis* BJ-18 in the seedlings of wheat. Wheat seedlings were grown in the presence of GFP-tagged *P. beijingensis* BJ-18 for 2 weeks. Images were taken with a fluorescent microscope. Excitation was at 488 nm. **(a)** Confocal image of the GFP-tagged *P. beijingensis* BJ-18 cells; **(b–e)** Colonization patterns in the root; **(f,g)** Colonization patterns in the stem; **(h)** Colonization patterns in the leaf. Bars represent 50 μm.

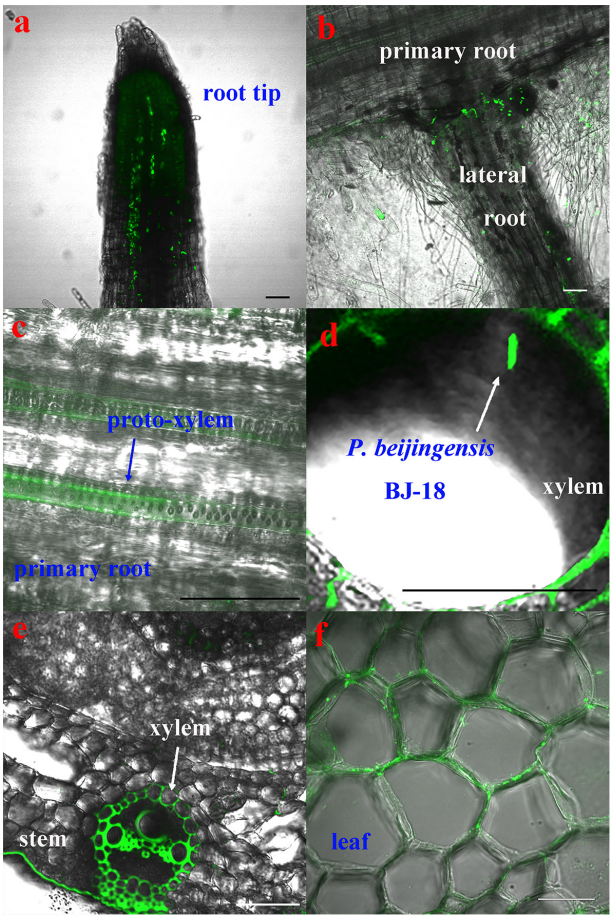

FIGURE 2 | Colonization of the GFP-tagged *P. beijingensis* BJ-18 in the seedlings of maize. Maize seedlings were grown in the presence of GFP-tagged *P. beijingensis* BJ-18 for 2 weeks. Images were taken with a fluorescent microscope. **(a–c)** Colonization patterns in the root; **(d,e)** Colonization patterns in the stem; **(f)** Colonization patterns in the leaf. Bars represent 50 μm.

Enhancement of GS and NR Activities of Plants by Inoculation With *P. beijingensis* 1-18

Compared to un-inoculated plant shoots and roots under low N (**Figures 8A,B**), GS activities were significantly increased in inoculated shoots of wheat (89.7%), maize (19.0%) and cucumber (46.9%) and in inoculated roots of wheat (45.0%), maize (85.5%) and cucumber (40.4%), but inoculation did not obviously enhance the GS activities of plants grown in high N soil.

As shown in **Figures 8C,D**, compared to un-inoculated plant shoots and roots grown in low N soil, NR activities were significantly enhanced in inoculated shoots of wheat (20.9%), maize (42.0%) and cucumber (28.9%) and in inoculated roots of wheat (28.9%), maize (66.2%) and cucumber (44.3%). In contrast, inoculation did not obviously enhance the NR activities of plants grown in high N soil.

Up-Regulation of Expression of N Uptake and N Assimilation Genes in Plants by Inoculation With *P. beijingensis*

In maize, the transcript levels of three genes (*ZmAMT1,1a*, *ZmAMT1,1b*, and *ZmNRT2.1*) involved in N uptake and five genes (*ZmNR*, *ZmNiR*, *ZmGS1-3*, *ZmGS1-4*, and *Zm CsGOGAT*) involved N metabolism were analyzed (**Figure 9**). Under low

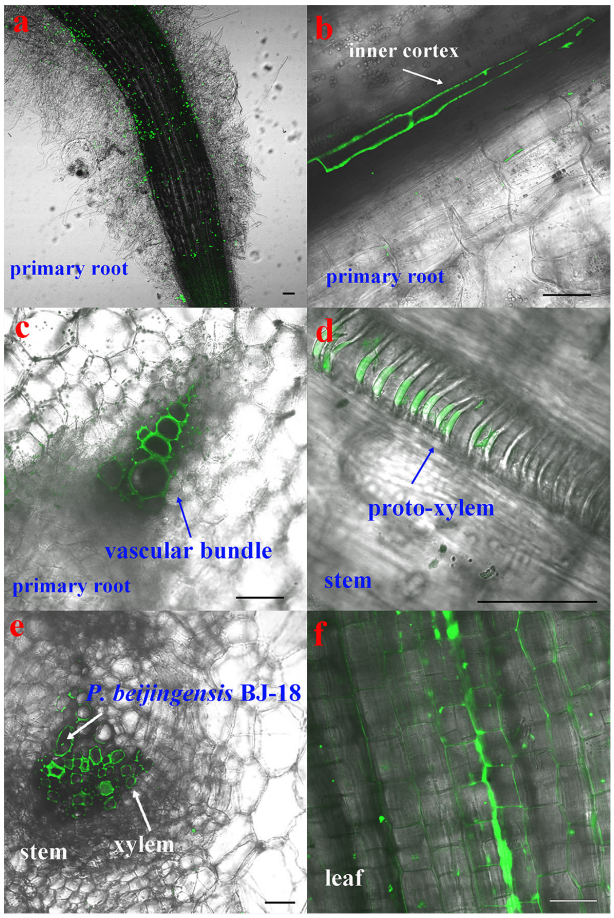

FIGURE 3 | Colonization of the GFP-tagged *P. beijingensis* BJ-18 in the seedlings of cucumber. Cucumber seedlings were grown in the presence of GFP-tagged *P. beijingensis* BJ-18 for 2 weeks. Images were taken with a fluorescent microscope. **(a–d)** Colonization patterns in the root; **(e)** Colonization patterns in the stem; **(f)** Colonization patterns in the leaf. Bars represent 50 μm.

N level, the transcript levels of *ZmAMT1,1a*, *ZmAMT1,1b*, and *ZmNRT2.1* were up-regulated by 1.83–3.93 folds in the inoculated shoots, while in inoculated roots, they were up-regulated 2.97–14.17 folds (**Figures 9A–C**), suggesting that inoculation promoted expression of plant N uptake genes. Similarly, inoculation significantly enhanced the transcript levels of *ZmNR*, *ZmGS1-3*, and *ZmGS1-4* in shoots by 18.46, 7.44, and 11.79 folds, respectively, and in roots by 5.63, 6.64, and 6.59 folds, respectively (**Figures 9D,F,G**). In contrast, inoculation did not obviously affect the transcript levels of these genes in maize shoots and roots under high N condition. However, inoculation did not affect the transcript levels of *ZmNiR* and *ZmGOGAT* under both low N and high N conditions (**Figures 9E,H**).

In wheat, the transcript levels of four genes (*TaAMT1.1*, *TaNRT1.1*, *TaNRT2.1*, and *TaNRT2.3)* involved in N uptake and four genes (*TaNR*, *TaNiR*, *TaGS1*, and *TaGOGAT)* involved N metabolism were analyzed. Similar to maize, the eight genes in the inoculated shoots of wheat in low N were up-regulated by 1.56–46.49 folds, while they were up-regulated 1.98–91.93 folds in inoculated roots (**Figure 10**). In contrast, inoculation did not obviously affect the transcript levels of these genes under high N condition.

In cucumber, the transcript levels of four genes (*CsAMT1*, *CsAMT3*, *CsNRT1.3*, and *CsNRT2.2*) involved in N uptake and five genes (*CsNR1*, *CsNR2*, *CsNiR*, *CsGS1*, and *CsGOGAT*) involved N metabolism were also analyzed. Similar to wheat and maize, the nine genes in the inoculated shoots of cucumber in low N soil were up-regulated 1.60–5.82 folds, while they were up-regulated 1.47–11.85 folds in inoculated roots (**Figure 11**). However, these effects of inoculated were weak when cucumber was grown in high N soil.

DISCUSSION

The N_2-fixing *Paenibacillus* strains have gained much attention due to their capacity of forming endospore to survive for long periods of time under adverse conditions (Grady et al., 2016). In this study, *P. beijingensis* BJ-18 was tagged by GFP and observation by laser confocal microscopy revealed that in seedlings of wheat, maize, and cucumber, bacterial cells could be found in the inner cortex and vascular bundle of roots and stems as well as within the leaves, suggesting *P. beijingensis* BJ-18 has similar invasion patterns in both monocotyledons and dicotyledons. The data indicated that *P. beijingensis* BJ-18 spread systemically from roots to stems and leaves of wheat, maize, and cucumber via xylem vessels. Therefore, *P. beijingensis* BJ-18 could be defined as a plant endophytic diazotrophic bacterium and it had a broad range of host plants. Similar colonization patterns were observed in the association of *P. polymyxa* WLY78 with wheat, maize, and cucumber (Hao and Chen, 2017) and in the association of *P. polymyxa* P2b-2R with lodgepole pine (a gymnosperm tree species) (Anand and Chanway, 2013). The colonization pattern of *P. beijingensis* BJ-18 was a little different form that of the associated diazotrophic *A. brasilense* Yu62 which colonized mainly on the surface of maize roots and only small part entered into maize tissues (Liu et al., 2003). These results indicated that on one side the diazotrophic *Paenibacillus* species/strains may fix N_2 inside plants and rapidly transfer the fixed product to plants, and on the other side they fix N_2 on the root surfaces and part of the fixed product may be remain in the soil.

In this study, the concentrations of *P. beijingensis* cells in inoculated plant tissues under high N and low N levels were determined by qPCR, with un-inoculated plant tissues as control. The results showed that the bacterial cell numbers were significantly higher in inoculated plant tissues grown under low N condition than those under high N condition, suggesting that soil N status controlled the concentration of bacterial cells in plants. Similar reports were found in sugarcane where high dose of N (ammonium nitrate) resulted in reduction of endophytic *Acetobacter diazotrophicus* concentration (Fuentes-Ramirez et al., 1999) and a higher number of endophytic diazotrophs were isolated from sugarcane plants under low N than under high N (de Oliveira et al., 2003). Moreover,

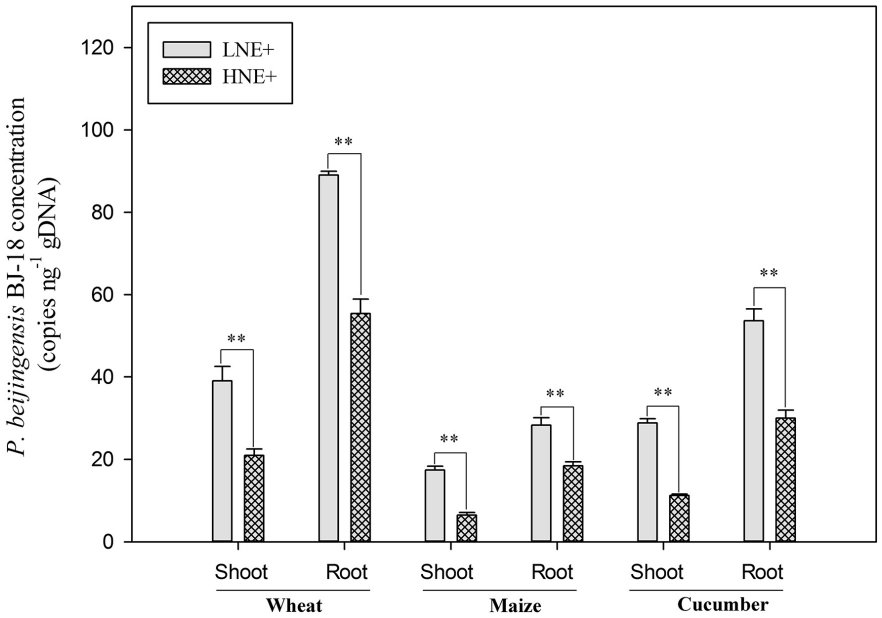

FIGURE 4 | The cell concentrations of *P. beijingensis* BJ-18 in the inoculated shoots and roots of wheat, maize, and cucumber seedlings in high N (HN) and low N (LN) levels. The concentrations of *P. beijingensis* BJ-18 are represented by *nifB* gene copies ng^{-1} total genomic DNA. Values are given as mean of three independent biological replicates, and single asterisks or double asterisks (* or **) indicate significant differences between HNE+ and LNE+ treatments determined by LSD at $P < 0.05$ or $P < 0.01$. The bars represent the standard error. LNE+ indicates plants grown in low N level of soils and inoculated with *P. beijingensis* BJ-18; HNE+ indicates plants grown in high N level of soils and inoculated with *P. beijingensis* BJ-18.

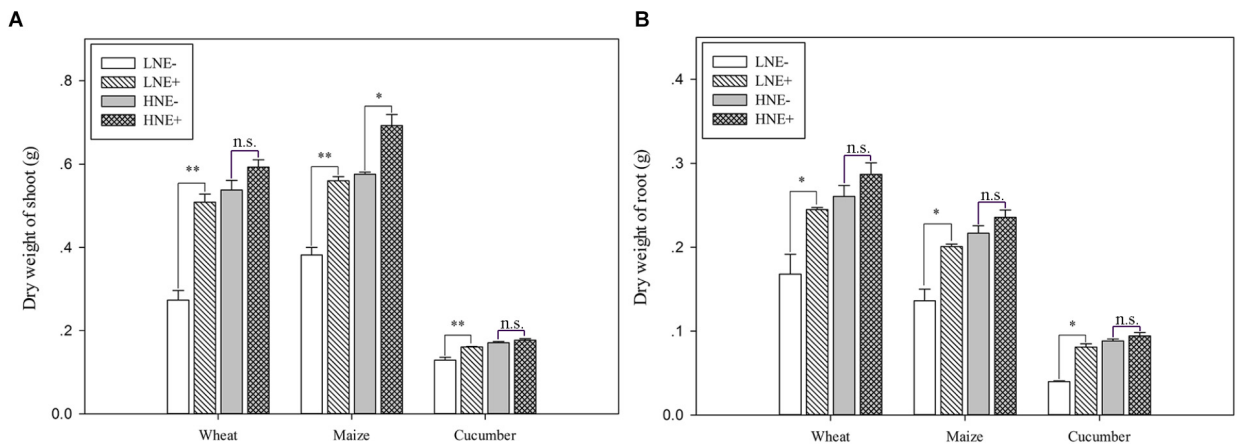

FIGURE 5 | Dry weight (DW) in shoots **(A)** and roots **(B)** of wheat, maize, and cucumber seedlings inoculated with *P. beijingensis* BJ-18 under high N (HN) and low N (LN) levels. Values are given as mean of three independent biological replicates, and single asterisks or double asterisks (* or **) indicate significant differences between inoculated (E+) and un-inoculated (E-) plants determined by LSD at $P < 0.05$ or $P < 0.01$. The bars represent the standard error. The wheat dry weight represents five seedlings per pot, and the dry weight of maize and cucumber represents one seedling per pot. LNE- indicates plants grown in low N level of soil and un-inoculated with *P. beijingensis* BJ-18; LNE+ indicates plants grown in low N level of soils and inoculated with *P. beijingensis* BJ-18; HNE- indicates plants grown in high N level of soil and un-inoculated with *P. beijingensis* BJ-18; HNE+ indicates plants grown in high N level of soils and inoculated with *P. beijingensis* BJ-18.

it was reported that the plant endogenous N status could also induce plant defense responses to regulate bacterial colonization (Carvalho et al., 2014). The increase in N compounds and amino acids (such as phenylalanine and hydroxyproline) was necessary to activate plant defense responses (Snoeijers et al., 2000). Amino acid transporters regulated by N status also have regulatory function in plant defense responses (Liu et al., 2010; Seifi et al., 2013). This study also showed that total N content and concentrations of NH_4^+ and NO_3^- in un-inoculated plants under high N were much higher than those under low N. This finding may explain why high N caused a decrease in the numbers of *P. beijingensis* cells in plants tissues.

Furthermore, this study investigated whether *nifH* gene was expressed in the inoculated plants. The expression levels of *nifH*

TABLE 2 | ^{15}N isotope enrichment determination of biological N_2 fixation rate in inoculated plants grown in soils containing high N and low N.

Treatments			δ15N value (versus at-air)		%Ndfa	
			High N	Low N	High N	Low N
Cucumber	Shoot	E-		10952 ± 1251a	3680 ± 261a	
		E+	7641 ± 727a	2656 ± 176b	25.4 ± 0.4b	27.8 ± 0.4a
	Root	E-	12926 ± 443a	4282 ± 738a	—	—
		E+	9457 ± 197b	2935 ± 503b	26.8 ± 1.1b	31.4 ± 0.9a
Wheat	Shoot	E-	6662 ± 1076a	3056 ± 299a	—	—
		E+	4596 ± 631a	1940 ± 171b	30.5 ± 1.8b	36.4 ± 0.6a
	Root	E-	6123 ± 316a	3573 ± 234a	—	—
		E+	4758 ± 274b	2614 ± 172b	22.3 ± 0.9b	26.9 ± 0.4a
Maize	Shoot	E-	5383 ± 434a	2222 ± 137a	—	—
		E+	4677 ± 296a	1756 ± 88b	12.9 ± 1.5b	20.9 ± 1.0a
	Root	E-	4523 ± 174a	2357 ± 292a	—	—
		E+	3885 ± 160a	1932 ± 235b	14.1 ± 0.4b	18.0 ± 0.3a

Values are given as mean ± SE of three independent biological replicates. Different letters indicates indicate significantly differences in δ15N value inoculated (E+) and un-inoculated (E-) plants according to the LSD test ($P < 0.05$); Different letters indicate significantly differences in %Ndfa between high N and low N according to the LSD test ($P < 0.05$); $\delta^{15}N$ value: percent atom excess ^{15}N; %Ndfa: percent N derived from atmosphere.

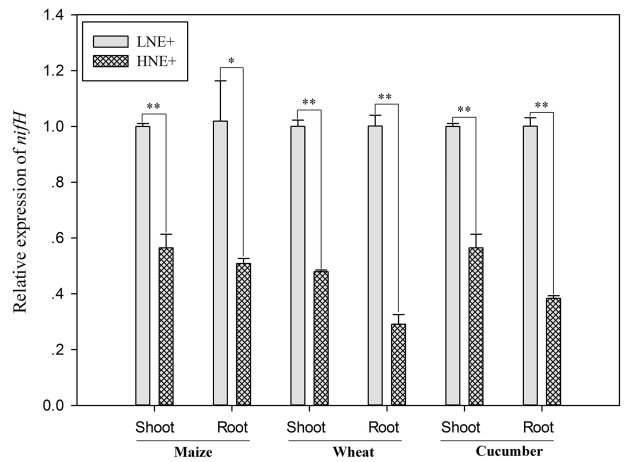

FIGURE 6 | The *nifH* transcript levels of *P. beijingensis* BJ-18 in shoots and roots of wheat, maize and cucumber seedlings inoculated with *P. beijingensis* BJ-18 under high N (HN) and low N (LN) levels. Values are given as mean of three independent biological replicates, and single asterisks or double asterisks (* or **) indicate significant differences between HNE+ and LNE+ treatments determined by LSD at $P < 0.05$ or $P < 0.01$. The bars represent the standard error. LNE+ indicates plants grown in low N level of soils and inoculated with *P. beijingensis* BJ-18; HNE+ indicates plants grown in high N level of soils and inoculated with *P. beijingensis* BJ-18.

in the inoculated plant shoots and roots under low N were significantly higher than those under high N, consistent with the concentrations of *P. beijingensis* cells. It was well known that *nif* gene expression was inhibited by high concentrations of NH_4^+ (Dixon and Kahn, 2004). GlnR mediated positive and negative regulation of *nif* gene expression in *P. polymyxa* WLY78 according to N availably (Wang et al., 2018). As mentioned above, there were high concentrations of NH_4^+ in plants under high N. Thus, *P. beijingensis* cells in plant tissues sensed the N signal and then regulated *nifH* expression according to change of N levels. The expression of *nif* gene of *Herbaspirillum seropedicae* in maize, sorghum, wheat, and rice plants was reported (Roncato-Maccari et al., 2003). Similar report was found that N fertilizer application inhibited *nifH* gene expression of the endophytic diazotroph in sugarcane leaves (Jia Hui et al., 2017).

An important metric to evaluate the role of diazotrophic bacteria is whether they can provide fixed N_2 to the host plant. The ^{15}N isotope dilution analysis has been widely applied to quantify BNF in non-legume plant species such as rice inoculated with *Herbaspirillum seropedicae* Z67 (James et al., 2002), wheat with *Azospirillum brasilense* Wa5 (Christiansenweniger and Vanveen, 1991), Kallar grass with *Azoarcus* sp. BH72 (Hurek et al., 2002) and maize with *P. polymyxa* P2b-2R (Puri et al., 2015). Here, ^{15}N isotope enrichment analysis method was used to estimate the contribution of BNF by *P. beijingensis* BJ-18 inoculation to plants. The BNF rates in the three different plants were higher under low N level than under high N level, indicating that BNF was affected by N levels. The data were consistent with the *nif* gene expression and the concentrations of *P. beijingensis* BJ-18 within the plant tissues under different N levels. Similar results were reported that sugarcane plants inoculated with diazotrophic strains gained higher N from BNF in the N-deficient soil (de Oliveira et al., 2003). This study also revealed that wheat gained the highest N from BNF under both low N and high N levels, followed by cucumber and then by maize, suggesting that BNF rate was related to host plant species. The shoots and roots of palm inoculated with *Bacillus sphaericus* UPMB-10 gained 13.2–13.4% N from BNF (Zakry et al., 2012), which was lower than that gained by inoculation with *P. beijingensis* BJ-18.

In this study, the effects of *P. beijingensis* BJ-18 inoculation on plant N uptake and metabolism were investigated. The

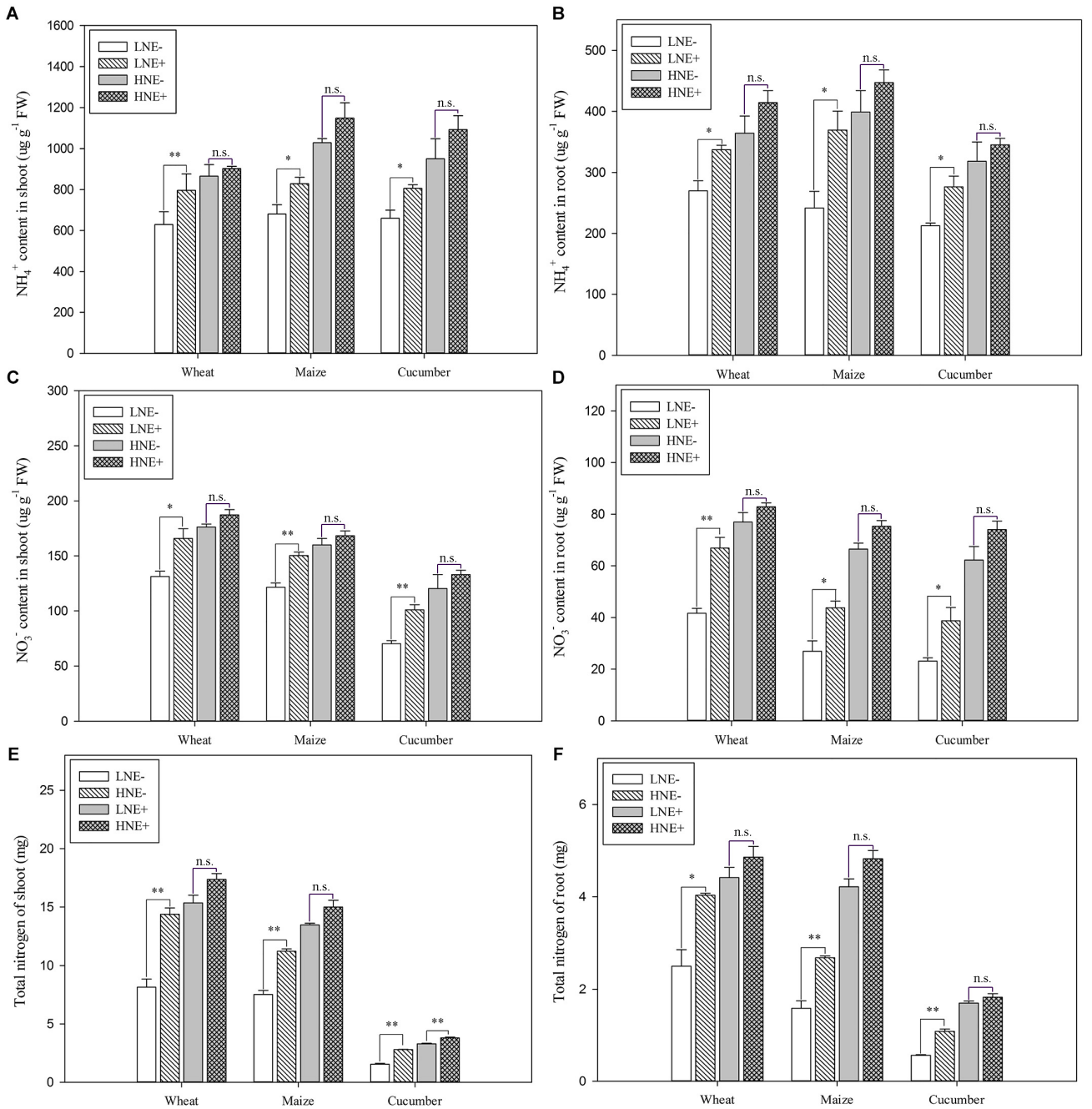

FIGURE 7 | Ammonium (NH_4^+) (**A**: shoot; **B**: root), nitrate (NO_3^-) (**C**: shoot; **D**: root) and total N (**E**: shoot; **F**: root) content in shoots and roots of wheat, maize and cucumber seedlings inoculated with *P. beijingensis* BJ-18 under high N (HN) and low N (LN) levels. Values are given as mean of three independent biological replicates, and single asterisks or double asterisks (* or **) indicate significant differences between inoculated (E+) and un-inoculated (E-) plants determined by LSD at $P < 0.05$ or $P < 0.01$. The bars represent the standard error. LNE- indicates plants grown in low N level of soil and un-inoculated with *P. beijingensis* BJ-18; LNE+ indicates plants grown in low N level of soils and inoculated with *P. beijingensis* BJ-18; HNE- indicates plants grown in high N level of soil and un-inoculated with *P. beijingensis* BJ-18; HNE+ indicates plants grown in high N level of soils and inoculated with *P. beijingensis* BJ-18.

concentrations of NO_3^-, NH_4^+ and total N were higher in roots and shoots of inoculated plants than in un-inoculated plants, and were higher in roots than in shoots. The positive effects were also controlled by soil N status. The results indicated that inoculation with *P. beijingensis* BJ-18 promoted plants to uptake NO_3^- and NH_4^+ from soil. The increased concentrations of NH_4^+ and total N in inoculated plants were at least partially resulted from BNF. Studies on the effects of diazotrophs on plant N uptake and metabolism were rare. However, inoculations with some endophytic fungi significantly

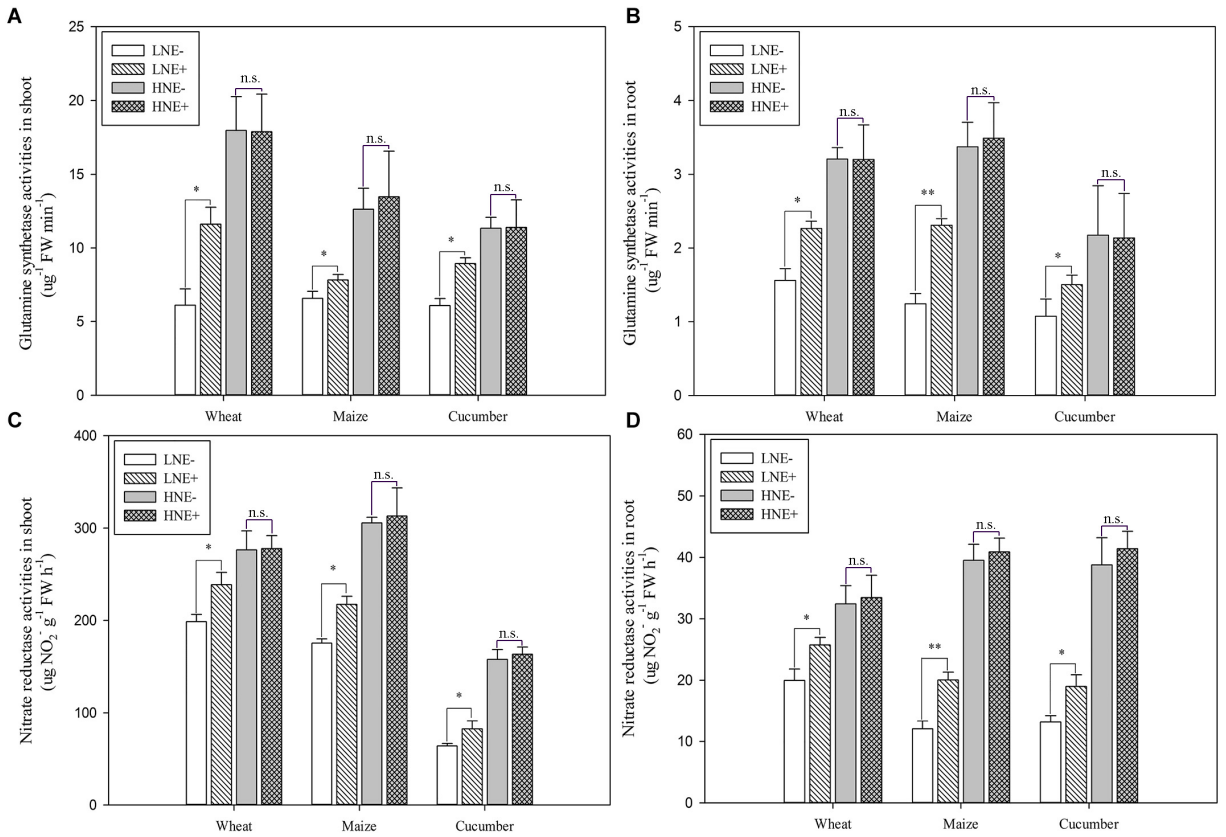

FIGURE 8 | Glutamine synthetase (GS) (**A**: shoot; **B**: root) and Nitrate reductase (NR) (**C**: shoot; **D**: root) activities in shoots and roots of wheat, maize, and cucumber seedling inoculated with *P. beijingensis* BJ-18 under high N (HN) and low (LN) levels. Values are given as mean of three independent biological replicates, and single asterisks or double asterisks (* or **) indicate significant differences between inoculated (E+) and un-inoculated (E-) plants determined by LSD at $P < 0.05$ or $P < 0.01$. The bars represent the standard error. LNE- indicates plants grown in low N level of soil and un-inoculated with *P. beijingensis* BJ-18; LNE+ indicates plants grown in low N level of soils and inoculated with *P. beijingensis* BJ-18; HNE- indicates plants grown in high N level of soil and un-inoculated with *P. beijingensis* BJ-18; HNE+ indicates plants grown in high N level of soils and inoculated with *P. beijingensis* BJ-18.

improved N accumulation and metabolism were observed in rice (Yang et al., 2014), sugar beet (Shi et al., 2009), and tall fescue (Lyons et al., 1990).

NO_3^- was mainly absorbed via NRT protein family members, and then transformed into NH_4^+ by NR. In this study, *P. beijingensis* BJ-18-inoculated plants showed significantly higher expression levels of *NRT* genes (*CsNRT1.3* and *CsNRT2.2* in cucumber; *TaNRT1.1*, *TaNRT2.1*, and *TaNRT2.3* in wheat; *ZmNRT2.1* in maize) in both shoots and roots under low N condition. Similar reports were found that under low N condition *NRT* genes were significantly up-regulated in rice inoculated with endophytic fungus *Phomopsis liquidambari* (Yang et al., 2014) and in tomato inoculated with diazotrophic *Enterobacter radicincitans* (Berger et al., 2013).

Higher NR activities were observed in the *P. beijingensis* BJ-18-inoculated plants. It was reported that inoculation with endophytic fungus *Plectosphaerella cucumerina* F11 greatly increased the activity of NR in sugar beet (Shi et al., 2009). To investigate whether the changes of NR activities in the *P. beijingensis* BJ-18- inoculated plants were closely related to differential expression of plant *NR* genes, the expression levels of *NR* genes were quantified. qRT-PCR analysis indicated that the expression levels of *NR* genes (*CsNR2* in cucumber, *TaNR* in wheat and *ZmNR* in maize) were significantly higher in inoculated plants than in un-inoculated plants under low N. It was reported that the endophytic fungus *Piriformospora indica* inoculation promoted N accumulation in *Arabidopsis* and tobacco seedlings by inducing the expression of *NR* (Sherameti et al., 2005). In contrast, endophytic fungus *Phomopsis liquidambari* inoculation significantly reduced NO_3^- concentration in rice shoots under low N condition, since the higher NR activity made more NO_3^- to be transformed into NH_4^+ (Yang et al., 2014).

This study demonstrated that *P. beijingensis* BJ-18-inoculated plants showed higher NH_4^+ concentration in roots and shoots, compared with those in un-inoculated plants. NH_4^+ was absorbed via AMT protein family members mainly, and then transformed into organic molecules by GS and GOGAT. GS is an important rate-limiting enzyme in NH_4^+ assimilation. The higher GS activities were observed in the *P. beijingensis* BJ-18-inoculated plants than those in un-inoculated plants. GS activities were closely related to soil N status. Transcript levels of

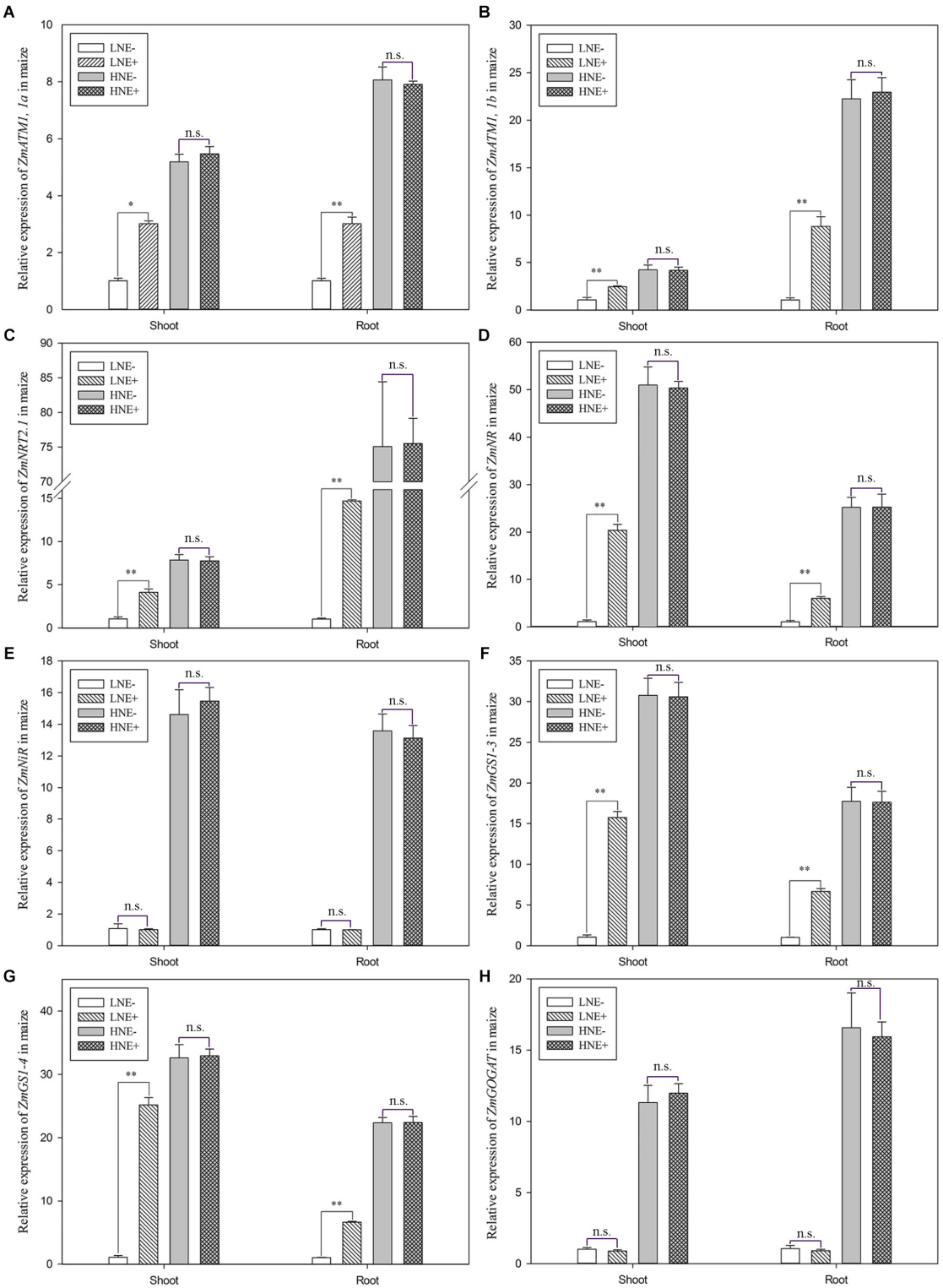

FIGURE 9 | The transcript levels of genes involved N uptake (**A**: *ZmAMT1, 1a*; **B**: *ZmAMT1, 1b*; **C**: *Zm NRT2.1*) and assimilation (**D**: *ZmNR*; **E**: *ZmNiR*; **F**: *ZmGS1-3*; **G**: *ZmGS-4*; **H**: *ZmGOGAT*) in shoots and roots of maize seedlings inoculated with *P. beijingensis* BJ-18 under high N (HN) and low N (LN) levels. Values are given as mean of three independent biological replicates, and single asterisks or double asterisks (* or **) indicate significant differences between inoculated (E+) and un-inoculated (E-) plants determined by LSD at $P < 0.05$ or $P < 0.01$. The bars represent the standard error. LNE- indicates plants grown in low N level of soil and un-inoculated with *P. beijingensis* BJ-18; LNE+ indicates plants grown in low N level of soils and inoculated with *P. beijingensis* BJ-18; HNE- indicates plants grown in high N level of soil and un-inoculated with *P. beijingensis* BJ-18; HNE+ indicates plants grown in high N level of soils and inoculated with *P. beijingensis* BJ-18.

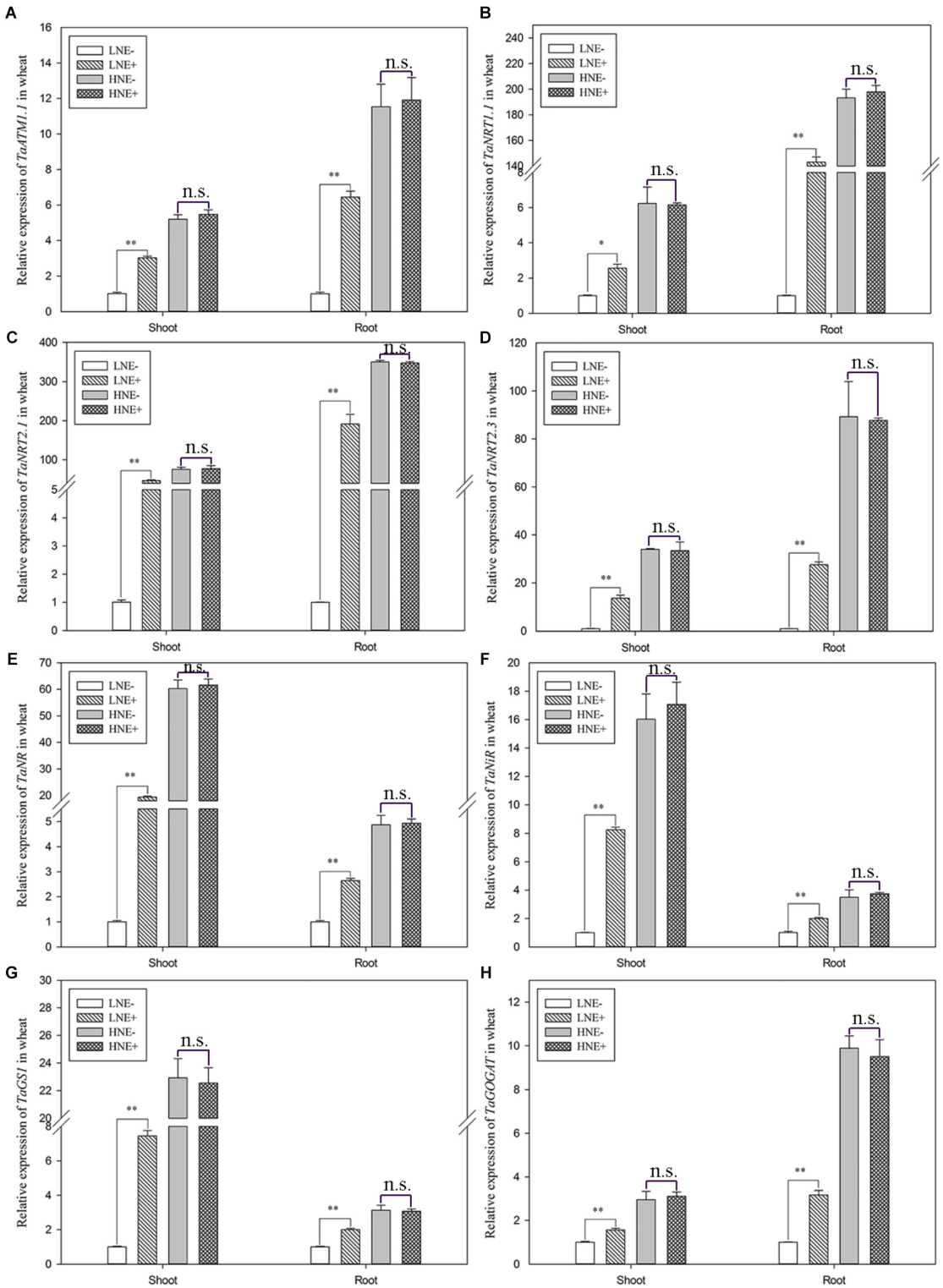

FIGURE 10 | Transcript levels of genes involved N uptake (**A**: *TaAMT1.1*; **B**: *TaNRT1.1*; **C**: *TaNRT2.1*; **D**: *TaNRT2.3*) and assimilation (**E**: *TaNR*; **F**: *TaNiR*; **G**: *TaGS1*; **H**: *TaGOGAT*) in shoots and roots of wheat seedlings inoculated with *P. beijingensis* BJ-18 under high N (HN) and low N (LN) levels. Values are given as mean of three independent biological replicates, and single asterisks or double asterisks (* or **) indicate significant differences between inoculated (E+) and un-inoculated (E-) plants determined by LSD at $P < 0.05$ or $P < 0.01$. The bars represent the standard error. LNE- indicates plants grown in low N level of soil and un-inoculated with *P. beijingensis* BJ-18; LNE+ indicates plants grown in low N level of soils and inoculated with *P. beijingensis* BJ-18; HNE- indicates plants grown in high N level of soil and un-inoculated with *P. beijingensis* BJ-18; HNE+ indicates plants grown in high N level of soils and inoculated with *P. beijingensis* BJ-18.

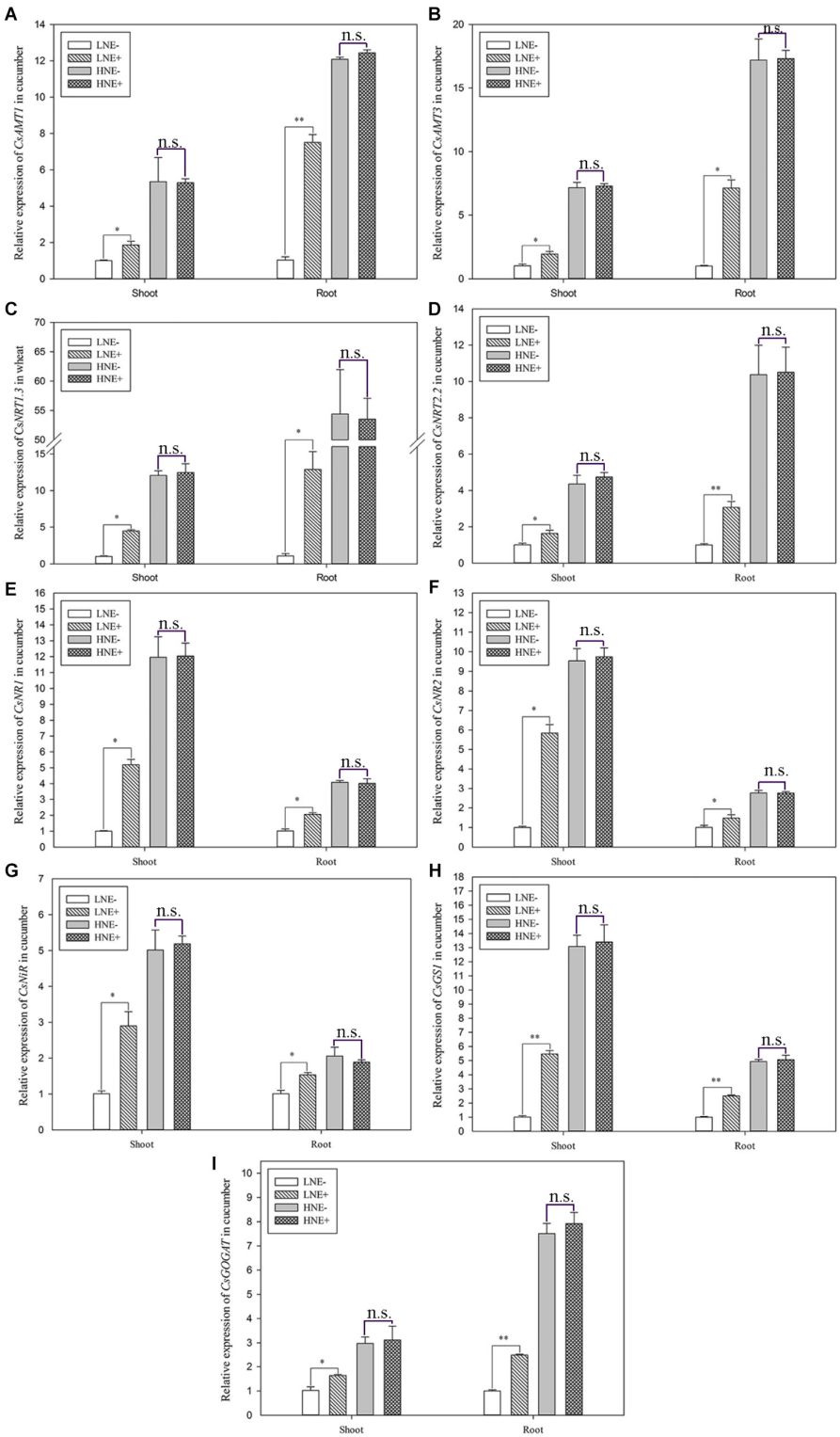

FIGURE 11 | The transcript levels of genes involved N uptake (**A**: *CsAMT1*; **B**: *CsAMT3*; **C**: *CsNRT1.3*; **D**: *CsNRT2.2*) and assimilation (**E**: *CsNR1*; **F**: *CsNR2*; **G**: *CsNiR*; **H**: *CsGS1*; **I**: *CsGOGAT*) in shoots and roots of cucumber seedlings inoculated with *P. beijingensis* BJ-18 under high N (HN) and low N (LN) levels. Values are given as mean of three independent biological replicates, and single asterisks or double asterisks (* or **) indicate significant differences between inoculated (E+) and un-inoculated (E-) plants determined by LSD at $P < 0.05$ or $P < 0.01$. The bars represent the standard error. LNE- indicates plants grown in low N level of soil and un-inoculated with *P. beijingensis* BJ-18; LNE+ indicates plants grown in low N level of soils and inoculated with *P. beijingensis* BJ-18; HNE- indicates plants grown in high N level of soil and un-inoculated with *P. beijingensis* BJ-18; HNE+ indicates plants grown in high N level of soils and inoculated with *P. beijingensis* BJ-18.

GS genes were also measured to confirm whether the changes of GS activities in plants caused by *P. beijingensis* BJ-18 inoculation were related to *GS* genes transcription. qRT-PCR indicated that the higher expression levels of *GS* genes (*CsGS1*, *TaGS1*, *ZmGS1-3*, and *ZmGS1-4*) in inoculated plant tissues than in un-inoculated ones under low N. Similarly, it was also reported that the expression levels of *GS* genes were also significantly higher in inoculated plants with the endophytic fungus *P. liquidambari* than in un-inoculated plants under low N (Yang et al., 2014).

This study demonstrated that inoculation with *P. beijingensis* BJ-18 promoted dry weight of plant roots and shoots grown under low N to be increased by 20.3–103.6%. The current results were in agreement with previous reports that inoculation with *P. beijingensis* BJ-18 increased wheat yield by 26.9% in field experiment (Shi et al., 2016) and increased tomato shoot length, fresh weight, and dry weight in the pot experiments (Xie et al., 2016).

The plant-growth-promoting rhizobacteria facilitate plant growth by several direct and indirect mechanisms. Direct mechanisms include P solubilization, N fixation and hormone (e.g., IAA, cytokinins and gibberellins) production. Indirect mechanisms include controlling phytopathogens by producing antibiotics or lytic enzymes (Glick, 2012). As mentioned above, *P. beijingensis* BJ-18 provided N for plants by BNF and thus promoted plant growth. Also, this bacterium may promote plant growth by producing IAA and antimicrobial compounds (Xie et al., 2016). Compared to *A. brasilense* Yu62 which produced high amount of IAA, *P. beijingensis* BJ-18 produced a little amount of IAA. Thus, IAA produced by *P. beijingensis* BJ-18 might be not the major factor promoting plant growth. Since the soil used in greenhouse study was not sterile, the BNF/plant growth promotion observed in this study could have been in part due to one or several indigenous microbes present in that soil. This study for the first time revealed that *P. beijingensis* BJ-18 promoted plants to uptake N from soil and enhanced gene expression and enzyme activities involved in N uptake and assimilation in plants. In addition to BNF, these endogenous changes in plants induced by *P. beijingensis* BJ-18 might be another major factor promoting plant growth.

CONCLUSION

This study demonstrated that *P. beijingensis* BJ-18 was an effective and endophytic diazotrophic bacterium which has similar colonization patterns in monocotylous and dicotyledonous plants. This bacterium promoted plant growth by direct mechanisms through BNF. Also, this bacterium might promote plant growth by indirect mechanisms through inducing endogenous changes in plants, including enhancement of N uptake and enzyme activities, and expression of N uptake and assimilation genes. The bacterial density within plant was closely related to the BNF efficiency and the endogenous changes in plants. However, the bacterial density, the BNF efficiency and the endogenous changes in plants during the association with *P. beijingensis* BJ-18 were controlled by the soil N status. These data suggested that successful colonization of *P. beijingensis* BJ-18 on plant was the first key step for this bacterium to promote plant growth by BNF and by inducing endogenous changes in plants. How soil N level affects bacterial colonization deserves further study.

AUTHOR CONTRIBUTIONS

YbL and SC designed and wrote the manuscript. YbL, YlL, HZ, and MW conducted the experiments.

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