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Incorporation of a DNA Sequence Encoding Green Fluorescent Protein (GFP) into Endophytic Diazotroph from Sugarcane and Sweet Potato and the Colonizing Ability of these Bacteria in *Brassica oleracea*

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Three endophytic bacterial strains (*Burkholderia* sp. no. 25, *Enterobacter* sp. no. 35 and *Klebsiella* sp. no. 38) isolated from sweet potato and sugarcane were examined for their ability to incorporate the gene encoding green fluorescent protein (GFP) via conjugation and electroporation. *Enterobacter* sp. no. 35 and *Klebsiella* sp. no. 38 were successfully tagged with the *gfp* gene by conjugation using pTn5kmgfpmut1. Fluorescence microscopic observation of *Brassica oleracea* inoculated with *gfp*-tagged endophytes revealed that *Enterobacter* sp. no. 35 and *Klebsiella* sp. no. 38 colonized the junctions between the lateral roots and the main roots.

Key words: *Enterobacter* sp., *Klebsiella* sp., conjugation, *Brassica oleracea*

Introduction

Endophytic bacteria are prokaryotic organisms that can reside in the internal portion of a host plant without triggering harmful reactions or disease symptoms.

It has been reported that some diazotrophic endophytes can colonize the interior of sugarcane[22,23]. Diazotrophic endophytes isolated and confirmed as nitrogen-fixing bacteria from sugarcane (*Saccharum officinarum* L.) include *Gluconacetobacter diazotrophicus, Herbaspirillum seropedicae* and *Herbaspirillum rubrisubalbicans*[4,6,9,22]. Some nitrogen-fixing bacteria have also been isolated from sweet potato (*Ipomoea batatas* L.)[1,2,5], and using the natural abundance of ^{15}N method, the contribution of biological nitrogen fixation (BNF) in sweet potato has been estimated at 26 to 44%[31].

Sweet potato and sugarcane are important crops in southern provinces of Japan. Investigation of the infection route and colonization of endophytes is very important in the practical use of biological nitrogen fixation (BNF). Reporter genes such as *lacZ*[3] and *gus*A[10] have been widely used in these studies. Recently, green fluorescent protein (GFP)[11] has become a valuable tool for research regarding plant-microbe interactions in living systems. It has been used to elucidate patterns of colonization and the spread of various bacteria[12,14,26,28,29]. In this study, we examined appropriate transformation methods for diazotrophic endophytes using electroporation and conjugation with three plasmids, pUTgfp×2[14,25,27], pFAJ1819[29] and pTn5Kmgfpmut1[19].

Brassica oleracea (broccoli) is a vegetable crop that requires lots of nitrogen fertilizer. To obtain a high yield, 270 kg N/hectare is needed[15]. Currently, there are no reports of the isolation of or infections by diazotrophic endophytes in this crop. We, therefore, examined the colonizing ability of *gfp*-tagged bacteria by inoculating them to broccoli.

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Materials and Methods

Bacterial strains

Endophytic bacterial strains belonging to 3 different genera isolated from sugarcane grown mainly on Miyako island and sweet potato grown on Miyakonojo have been investigated for diazotrophic characteristics by our group (Unpublished). Previously isolated endophytic bacterial strains (*Burkholderia* sp. no. 25 from *Ipomoea batatas* cv. Shiroyutaka, *Enterobacter* sp. no. 35 from *Saccharum officinarum* cv. Ni15 and *Klebsiella* sp. no. 38 from *Saccharum officinarum* cv. NiF8) belonging to 3 different genera were used in this study. The plant samples from which the endophytes were isolated, were surface sterilized with 70% ethanol for 5 min followed by 3% hydrogen peroxide for 30 sec and were thoroughly washed with sterile distilled water. After sterilization, samples were crushed in a mortar and the juice extract from crushed 10-g samples was mixed with 1 ml of 0.85% sterile saline. Then, 100 µl of the mixture was transferred into a test tube containing LGIP[23] semi-solid medium. The inoculated test tubes were incubated at 28°C for 6 days, after which 10% of the tube's head atmosphere was replaced with acetylene gas. Test tubes were further incubated for 24 hours. The concentration of ethylene was determined with Shimadzu GC8A gas chromatograph. From the acetylene reduction assay (ARA)—positive tubes, the endophytic bacteria were isolated by streaking a loop of the semi-solid agar onto solid agar LGIP plates. Single colonies were transferred to a semi-solid LGIP medium and the N_2-fixing bacteria were identified by ARA. These bacteria were tentatively identified by using the 16S rRNA gene sequence.

PCR amplification and sequencing of the 16S rRNA gene

Cell lysate from each isolate for use as the PCR template was prepared by the method of Hiraishi *et al.*[17] with slight modifications[24]. The PCR was performed with Ex *Taq* DNA polymerase (TaKaRa Bio Inc., Otsu, Japan). The 16S rRNA gene was amplified using the 20-mer forward primer 16S rDNAF (5'-AGAGTTTGATCCTGGCTCAG-3') and the 19-mer reverse primer 16S rDNAR (5'-GGCTACCTTGTTACGACTT-3'). The 16S rRNA gene fragment were amplified with 30 cycles of PCR (94°C 1 min, 50°C 1 min, 72°C 1 min). The PCR products were purified from an agarose gel after electrophoresis and were used as a template for direct sequencing using a Big Dye Terminator Ver 3.1 Cycle Sequencing Kit (PE. Applied Biosystems, Tokyo, Japan). The primers 16S rDNAF and 16S rDNA520R (5'-GTATTACCGGGCTGC-3') were used for partial sequences.

The 16S rRNA gene fragments were sequenced with 25 cycles of reaction (94°C 30 sec, 55°C 15 sec, 60°C 4 min). The sequences of the PCR product were determined with the primers directly by using an ABI PRISM 310 genetic analyzer (PE. Applied Biosystems, Tokyo, Japan). The BLAST search system available in the DDBJ DNA database was used to compare the resulting sequences with those identified in the DDBJ DNA database. The Unweighting Pair Group Method using the arithmetic Average (UPGMA) and GENETYX-MAC ver. 10.1 (GENETYX Corp, Tokyo, Japan) were used to construct a phylogenetic tree.

Database accession numbers for sequences

The 16S rRNA gene sequences determined in this study have been deposited under DDBJ accession numbers AB256514 to AB256516.

Fluorescent labeling of diazotrophic bacteria by electroporation

To confirm the endophytic features of the isolated endophytic bacteria, we incorporated the DNA sequence encoding the *gfp* gene into them. Three plasmids, pTn5Kmgfpmut1, pFAJ1819 and pUTgfp×2, were used in this study. pTn5kmgfpmut1 and pFAJ1819 contained the *gfp* minitransposon and expressed the *gfp* gene downstream of the constitutive promoter *pnpt2*. pUTgfp×2 contained the constitutive promoter *PbsA*. pTn5kmgfpmut1 carried one copy of the *gfp* gene and the kanamycin-resistance gene, whereas pFAJ1819 and pUTgfp×2 carried two tandem copies of the *gfp* gene and the kanamycin-resistance gene.

Each strain of endophytic bacterium was cultured in 2 ml of Luria-Bertani (LB) medium. A 100-µl aliquot of the subculture was transferred into 100 ml of LB medium and incubated at 30°C with shaking until the exponential phase. The cells were chilled on ice and harvested by centrifugation at 3000×g for 15 min at 4°C. Each pellet was then washed twice with 100 ml of cold sterilized distilled water, resuspended in 500 µl of cold sterile water with 10% glycerol, dispensed in 100-µl aliquots, and stored at −80°C prior to use. The competent cells were thawed on ice, and purified *gfp* delivery plasmids (200 ng) were added to the cells and mixed quickly. The mixture was incubated on ice for 15 min, transferred into a sterile pre-chilled cuvette (interelectrode gap: 0.2 cm), and placed in a Gene Pulser II apparatus equipped with a Pulse Controller (BioRad Laboratories, Tokyo, Japan). The electroporation unit was set at the following values: 12.5 kV/cm, 25 µF and 200 Ω. Following the

pulsing, the cells were immediately diluted with 1 ml of LB medium, transferred into a sterilized tube, and incubated at 30°C for 3–4 h. From each tube, 100 µl was plated onto LGIP agar containing 50 µg of kanamycin per ml. After the plates were incubated at 30°C for 12 h, the green fluorescence colonies were examined under a NIKON Eclipse E600 fluorescence stereomicroscope with a B-2A filter.

Fluorescent labeling of diazotrophic bacteria by conjugal transfer through mating

Each strain of endophytic bacterium, and the donor strains *Escherichia coli* s17-1λpir containing pTn5Kmgfpmut1, *E. coli* s17-1λpir containing pFAJ1819, and *E. coli* cc118λpir containing pUTgfp×2 were incubated separately in 2 ml of LB medium for 12 h at 37°C with shaking. The bacteria were harvested by centrifugation at 3000×g for 10 min at 25°C. Each pellet was then washed in saline and resuspended in 30 µl of saline. The cell suspension was mixed at an endophytic bacteria-to-*E. coli* ratio of 1:1, and the mixture was spotted onto LB agar plates. The plates were allowed to dry for 5 min, and then incubated for 24 hours at 28°C. The cell mixtures growing on LB agar plates were scraped off and resuspended in 1 ml of saline. A dilution series of the cell suspension was made and plated onto BIND (brilliant green, inositol, nitrate and deoxycholate)[21] agar plates containing 10 µg of kanamycin per ml of medium for *Klebsiella* and onto GMM (general minimal medium)[8] agar plates containing 10 µg of kanamycin per ml of medium for the other bacterial strains. The plates were incubated for 24 hours. The colonies were examined for green fluorescence under a Nikon Eclipse E600 fluorescence stereomicroscope with a B-2A filter.

Acetylene reduction activity of bacterial cultures

The nitrogen-fixing activity of the bacterial cultures was examined by conducting an acetylene-reduction assay in a 45-ml test tube containing 15 ml of LGIP semi-solid culture medium. Each strain of endophytic bacterium was cultured in 2 ml of LB broth. The bacterial suspension (0.1 ml) was transferred onto LGIP semi-solid medium and incubated for 5 days at 30°C. Acetylene gas was injected into the head atmosphere of the test tubes (30 ml) to a final concentration of 10% (vol/vol). The tubes were further incubated for 8 h at 30°C. The amount of ethylene in the headspace was determined using a Shimadzu GC-8A gas chromatograph equipped with a flame ionization detector and a Porapack N column (Shimadzu, Kyoto, Japan).

Condition for Plant growth and inoculation

Seeds of *Brassica oleracea* cv. Ryokurei were surface-sterilized with 70% ethanol for 1 min followed by 2% sodium hypochlorite for 15 min, and were then washed three

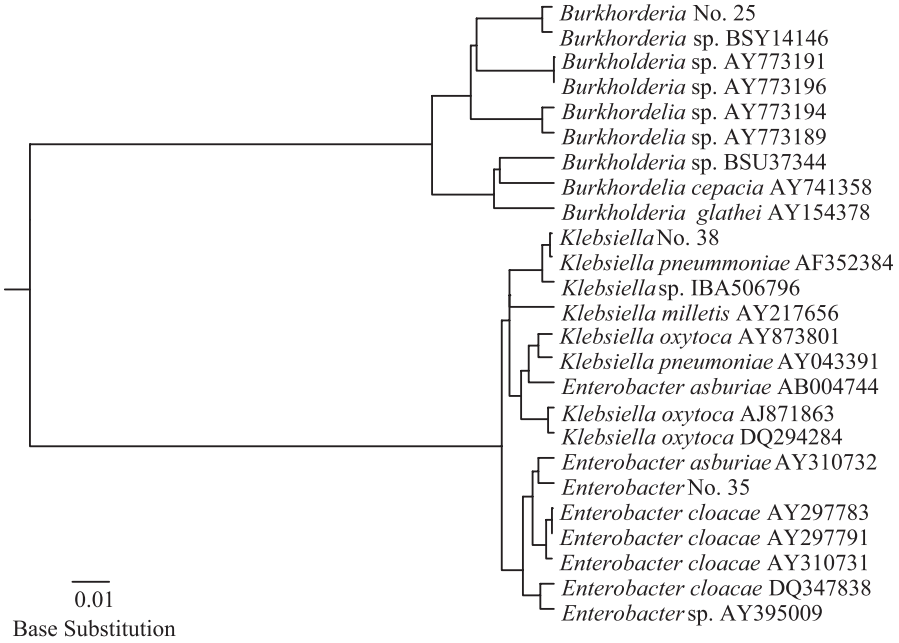

Fig. 1. Phylogenic tree of isolated strains and some control strains. The tree was generated with UPGMA. DNA sequences of some control strains were obtained from the DDBJ database.

Table 1. Relationship between plasmid type and method of introduction of the plasmid carrying the *gfp* gene

Organism	Introduction of the plasmid carrying the *gfp* gene					
	Electroporation			Conjugation		
	pUTgfp×2	pFAJ1819	pTn5Kmgfpmut1	pUTgfp×2	pFAJ1819	pTn5Kmgfpmut1
Burkholdelia sp. no. 25	×	×	×	×	×	×
Enterobacter sp. no. 35	×	×	×	×	×	○
Klebsiella sp. no. 38	×	×	×	×	×	○
Herbaspirillum sp. B501	○	—	—	—	—	—

Symbols: ○, introduced; ×, not introduced; —, not examined.

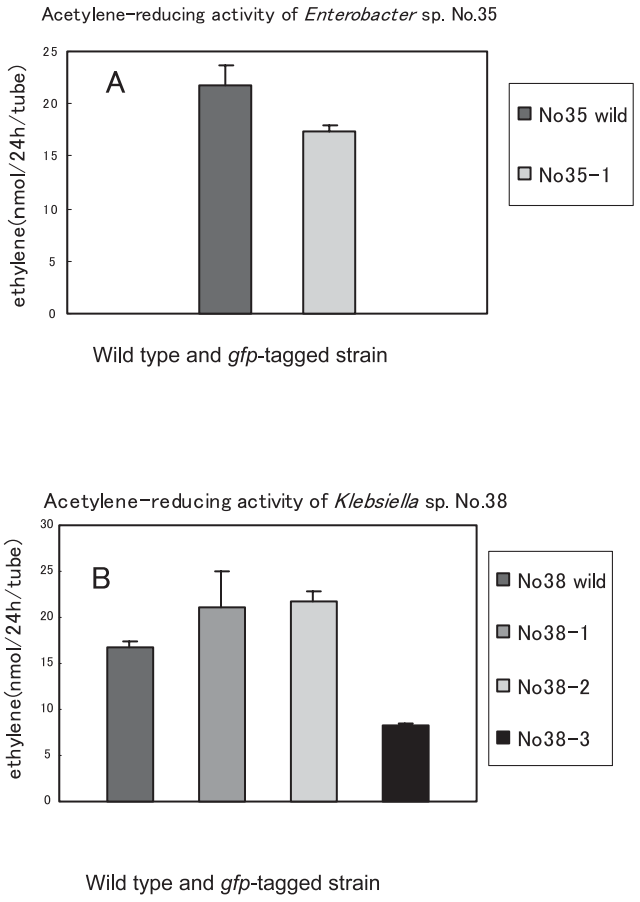

Fig. 2. Acetylene-reducing activity of the parent strains and transformants of *Enterobacter* sp. no. 35 and *Klebsiella* sp. no. 38. Comparison of the acetylene-reducing activity of the parent strain and some transformants of *Enterobacter* sp. no. 35 after *gfp*-gene tagging (A), and the parent strain and some transformants of *Klebsiella* sp. no. 38 after *gfp*-gene tagging (B).

times in sterilized distilled water with shaking. The seeds were germinated aseptically in a plastic container filled with vermiculite containing 40 ml of MS medium. The plants were kept at 28°C under a photoperiod of 16 h light. Each type of *gfp*-tagged bacterium was grown in 100 ml of LB broth, harvested at the exponential phase, and centrifuged at 3000×g for 10 min at 25°C. The supernatant was discarded and the cell pellet was resuspended in 20 ml of additional MS nutrient solution supplemented with 0.5 mM KNO_3. Ten-day-old seedlings were inoculated with *gfp*-tagged endophytic bacteria (10^{10} bacterial cells per seedling). The plants were allowed to grow for 7 days under the conditions described above.

The plants were harvested 7 days after the inoculation and washed with sterilized distilled water, surface-sterilized with 70% ethanol for 30 sec followed by 2% sodium hypochlorite for 2 min, and finally washed three times with sterilized distilled water. The stems were transversally sectioned using a microslicer (DTK1000, Dosaka EM Co., Ltd., Japan). The colonization patterns of the inoculated endophytes were observed under a Nikon Eclipse E600 fluorescence stereomicroscope with a B-2A filter (Ex 450–490).

Results and Discussion

Characterization of nitrogen-fixing bacteria

The diazotrophic isolates were closely related to the genera *Burkholderia*, *Enterobacter* and *Klebsiella*. Isolate no. 25 clustered close to *Burkholderia*, while Isolates no. 35 and no. 38 clustered close to *Enterobacter* and *Klebsiella*, respectively. (Fig. 1). Endophytic bacteria, *Pantoea agglomerans* and *Klebsiella oxytoca*, have been isolated from sweet potato previously[1,5]. However, no *Burkholderia* strain has ever been isolated from sweet potato. The endophytic bacteria *Enterobacter aerogenes* and *Klebsiella pneumoniae* have been isolated from sugarcane[20].

Tagging the bacterial strains with the gfp gene

We tried two methods of gene transformation in our en-

dophytes, electroporation and conjugation. The incorporation of the *gfp* gene by electroporation was not successful. None of the three strains examined was transformed, as shown in Table 1. To check our methodology, we used *Herbaspirillum* sp. B501[14] to incorporate the *gfp* gene by electroporation. This strain has been previously tagged with *gfp* using this method[14]. We succeeded in incorporating the *gfp* gene into *Herbaspirillum* sp. B501 using the plasmid pUTgfp×2.

Enterobacter sp. no. 35 and *Klebsiella* sp. no. 38 were successfully tagged with the *gfp* gene by conjugation with *E. coli* s17-1λpir containing pTn5kmgfpmut1 (Table 1). However, we did not succeed in incorporating the *gfp* gene using the other plasmids (pUTgfp×2 and pFAJ1819) by the same method. The growth and ARA activity of the transformants that showed strong *gfp* fluorescence were compared with those of their respective parent strains. All the *gfp*-tagged strains had almost the same growth curve as their respective parent strains. Based on their ARA, *Enterobacter* sp. no. 35-1 and *Klebsiella* sp. no. 38-2 were selected for the examination of colonizing ability in plant tissues of *Brassica oleracea* (Fig. 2).

Electroporation is a relatively difficult method of plasmid transformation which requires appropriate bacterial growth conditions, a proper composition of the electroporation solution, precise electroporation conditions (field strength, resistance, etc.), a proper size, concentration and purity of the DNA used for the transformant, and appropriate conditions for selecting the transformant[13]. The plasmids pUTgfp×2, pFAJ1819 and pTn5Kmgfpmut1 are all based on pUT[16]. Nevertheless, pTn5Kmgfpmut1 was the only plasmid which worked. Perhaps the lack of a helper strain such as *E. coli* HB101[3], which boosts the transformation efficiency, contributed to the failure. The helper strains normally used for triparental mating contain a *tra* gene, which mediates transformation[3,30]. In this study, we adopted biparental mat-

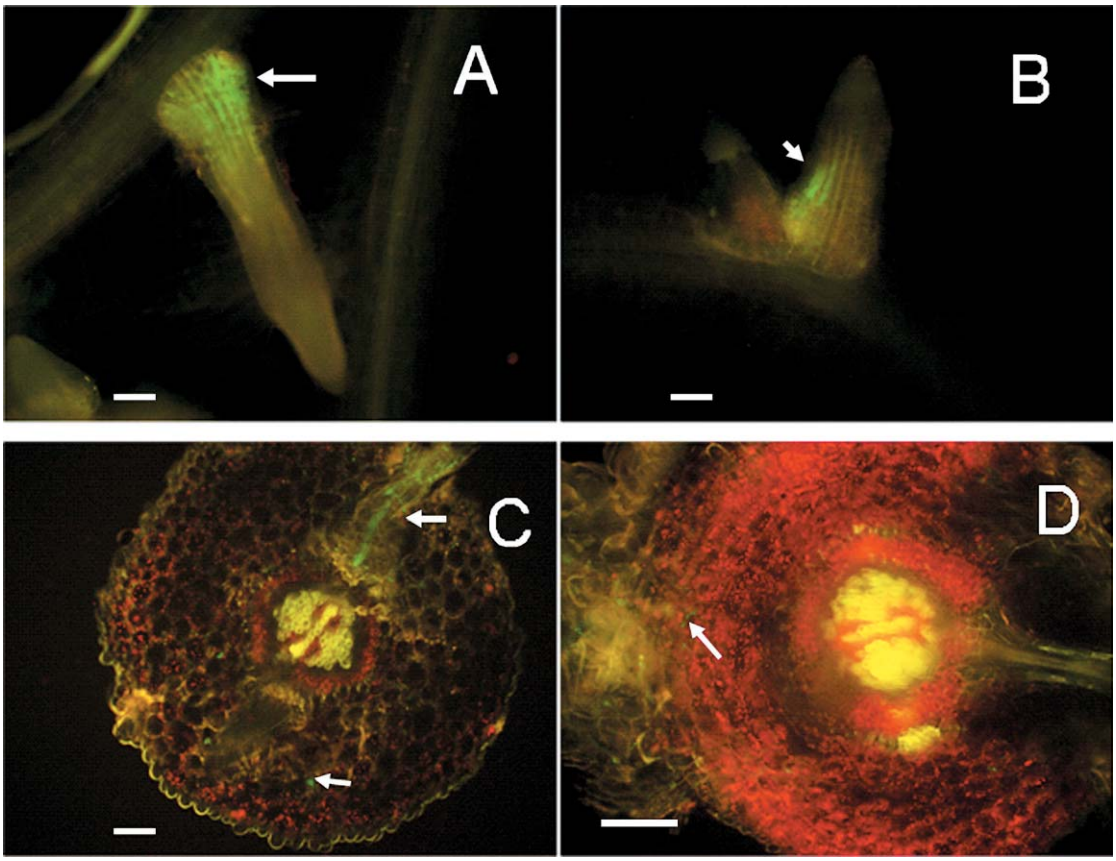

Fig. 3. Fluorescence micrographs showing the colonization of *Brassica oleracea* by endophytic diazotrophs 7 days after inoculation. Colonization of lateral roots at the junction with the main roots by *gfp*-tagged *Enterobacter* sp. no. 35-1 (A) and *Klebsiella* sp. no. 38-2 (B). Transversal sections of the basal stem showing the colonization of the cortex and lateral root stele by *Enterobacter* sp. no. 35-1 (C) and the colonization of the cortex by *Klebsiella* sp. no. 38-2 (D). Bar=10 μm (A to D).

ing, and succeeded in incorporating the *gfp* gene into three strains. However, the conjugation method of incorporating *gfp* must be optimized further for different diazotrophic endophytes.

Colonization and localization of gfp-tagged strains in plant tissues

Endophytic diazotrophs may have broad host compatibility, for example, an isolate from *Zea mays*, *H. seropedicae* could colonize and fix N_2 in *Oryza sativa*[7]. Similarly, we conducted an experiment in which *Enterobacter* sp. no. 35 and *Klebsiella* sp. no. 38 were transferred into *Brassica oleracea*, a non-host plant. Fluorescence microscopy showed that *Enterobacter* sp. no. 35-1 and *Klebsiella* sp. no. 38-2 successfully colonized the roots and the basal stem tissues of *Brassica oleracea*. *Enterobacter* sp. no. 35-1 colonized the junctions between the lateral roots and the main roots (Fig. 3A). Transversal sections showed that the bacteria colonized the base of the lateral roots (Fig. 3B). *Klebsiella* sp. no. 38-2 also colonized the stele of the lateral roots (Fig. 3C) and localized to the cortex area of the basal stem (Fig. 3D). No colonization was observed in the upper stem and the leaves by any of the 3 strains. This might be due to the inability of these bacteria to colonize these parts. Another reason may be the short inoculation period (7 days) which might not be long enough for these bacteria to move into the upper parts and colonize them. According to some, endophytic bacteria enter the root where lateral roots emerge[10,18]. This may be the point of entry in the roots of *Brassica oleracea* since the bacteria clearly seemed to colonize the base of lateral roots. No endophytic diazotrophic isolates from *Brassica oleracea* have ever been reported. The presence of bacterial colonies where the lateral roots emerge indicates that these are possible sites of entry by bacteria which later invade the internal tissues and penetrate into the stele area. The infection of lateral roots and the presence of bacterial colonies between cell layers in the cortex demonstrate the ability of the bacteria to penetrate deep into *Brassica oleracea*. The results indicate that endophytic diazotrophs can indeed colonize *Brassica oleracea* and are probably able to fix nitrogen. Therefore, the N^{15} absorption rate must be experimentally estimated to verify the possibility of endophytic N_2 fixation in *Brassica oleracea*.

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