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Colonization study of gfp-tagged Achromobacter marplatensis strain in sugar beet

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This study details the introduction of a gfp marker into an endophytic bacterial strain (*Achromobacter marplatensis* strain 17, isolated from sugar beet) to monitor its colonization of sugar beet (*Beta. vulgaris* L.). Stability of the plasmid encoding the gfp was confirmed *in vitro* for at least 72 h of bacterial growth and after the colonization of tissues, under nonselective conditions. The colonization was observed using fluorescence microscopy and enumeration of culturable endophytes in inoculated sugar beet plants that grew for 10 or 20 days. gfp-Expressing strains were re-isolated from the inner tissues of surface-sterilized roots and stems of inoculated plants, and the survival of the *Achromobacter marplatensis* 17:gfp strain in plants 20 days after inoculation, even in the absence of selective pressure, suggests that it is good colonizer. These results also suggest that this strain could be a useful tool for the delivery of enzymes or other proteins into plants. In addition, the study highlights that sugar beet plants can be used effectively for detailed *in vitro* studies on the interactions between *A. marplatensis* strain 17 and its host, particularly if a gfp-tagged strain of the pathogen is used.

Keywords: sugar beet, endophytes, green fluorescent protein, colonization

Introduction

Endophytic bacteria are defined as bacteria that can be isolated from surface-disinfected plant tissues or extracted from within plants and that are not observed to harm the host plants (Mcinroy and Kloepper, 1995). Endophytic bacteria ubiquitously inhabit most plant species and have been isolated from a variety of species (Lodewyck *et al.*, 2002). They span a range of bacterial phyla and have been reported to have important roles in determining plant yield, and promoting plant growth, health, and protection by means similar to plant growth-promoting rhizobacteria (PGPR) (Lodewyck *et al.*, 2002; Ryan *et al.*, 2008; Shi *et al.*, 2008; Wu *et al.*, 2014). Some endophytes show resistance to heavy metals and degrade organic compounds in the plant-soil-water niche and, thus, also have an important role in pollution control and phytoremediation (Newman and Reynolds, 2005). Therefore, a better understanding of endophytic bacteria could help to elucidate their function and potential role more effectively in developing sustainable systems of crop production.

Although healthy roots of sugar beet have been shown to harbor endophytic bacteria (Jacobs *et al.*, 1985), little is known about the distribution of these microorganisms during the growing season. Endophytes occupy microniches within plant tissues and some have been found to be PGPE (plant growth promoting endophytes). These endophytes are unaffected by competition from other microorganisms or conditions in the rhizosphere. As a result, a new approach has been developed in recent years that involves treating seeds and seedlings with PGPE, and can be an alternative to fertilizer use. Thus, inoculation of pepper with endophytic *Pseudomonas fluorescens* significantly increased the fresh weight, height, and stem diameter of treated plants (Lucas-Garca *et al.*, 2003).

In a previous study, 251 endophytic bacterial isolates were extracted from sterilized tissues from a widely planted sugar beet cultivar (*Beta vulgaris* L. *var. saccharifera*) variety Xintian 18 in a semi-arid region in east Urumuqi, Xinjiang, China. *Achromobacter marplatensis* strain 17 was isolated from sugar beet. This bacterial strain promoted sugar beet seedling growth following seed inoculation by seed dipping (Shi *et al.*, 2014). Plant height and dry weight of sugar beet increased by 19% and 69%, respectively, compared with controls. Bacterial strain 17 exhibited the ability to increase the absorption of N, P, K, and Mg elements from the soil and increased the content of vitamins B and C, and protein within beet plants (Shi *et al.*, 2014). In addition, the bacterial strain also produced a phytohormone (auxin), which resulted in the production of nearly twice as much IAA as that produced by strain 17, and was also able to solubilize phosphates. The concentration of dissolved P in the medium was 180.5 mg/L after 4 days of incubation (Shi *et al.*, 2014). In field experiments, strain 17 significantly increased the content of sucrose, fructose, and the yield of the sugar beet. The growth-promoting properties of strain 17 indicates that this isolate merits further investigation in terms of its symbiosis with sugar beet and its potential application in agriculture.

By studying the colonization, expansion, and distribution of endophytic bacteria in plants, it is possible to study and elucidate the mechanism behind the plant growth-promo-

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ting abilities of endogenous bacteria. However, there has been a lack of direct and effective technologies with which to perform such studies, which has restricted the further development of this field. It is difficult to directly and accurately distinguish antibiotic markers and radioactive isotopes traditionally used in labeling technology. There are also limitations to the use of such approaches. However, the development of luminescent gene marker techniques has provided a sensitive and reliable research method for the real-time, *in situ* monitoring of the ecological distribution of microorganisms in the environment as well as the study of the mechanisms behind the interaction between microorganisms and their hosts (Qazi *et al*., 2001). Among them, the green fluorescent protein (gfp) system with small genes (approximately 800 bp) (Cormack *et al*., 1996), high sensitivity (detection of single cell), good stability, real-time, *in situ* monitoring and other advantages, has been widely used in the study of microorganisms and their interactions with their environment and hosts, including gene expression regulation (Errampalli *et al*., 1999).

The first step in colonization studies is the assessment of colonization efficiency, which requires the precise identification of the inoculum. A simple method of tracking bacteria is the introduction of antibiotic resistance markers; however, there are many reports of strains losing their antibiotic-resistant phenotype (Nairn and Chanway, 2002). To address this problem, the addition of a second label, such as the *gfp* gene offers an interesting opportunity for ecology studies. This gene does not require any exogenous substrates for its expression and it allows the temporal and spatial tracking of the bacteria in plant tissues (Valdivia and Falkow, 1997; Errampalli *et al*., 1999; Compant *et al*., 2005).

The aim of the current study was to evaluate the colonization patterns of one gfp-tagged strain in sugar beet. The shuttle vector pgfp4412 was transformed into *A. marplatensis* strain 17, and its colonization of sugar beet was studied using fluorescence microscopy.

Materials and Methods

Bacterial strains, plasmids, and culture conditions

Achromobacter marplatensis strain 17 (from the Culture Collection of Plant Research International), CGMCC No. 8192, was used in the current study. The strain was grown on tryptone soy agar (TSA) medium (per L: tryptone 10 g, yeast extract 5 g, NaCl 5 g, pH 7.5, and agar 15 g) at 25°C for 48 h.

The main source of nitrogen for the endophytic PGPR strain 17 in the current study included, but was not limited to, peptone and yeast powder. The main source of carbon included, but was not limited to, sucrose, glycerol, mannitol, and maltose. Inorganic components included, but were not limited to, potassium chloride, sodium chloride, sodium dihydrogen phosphate, dipotassium hydrogen phosphate, tricalcium phosphate, calcium chloride dihydrate, magnesium sulfate heptahydrate, and ferrous sulfate heptahydrate. PGPR fermentation can be carried out at 20–37°C, pH 5.5–9.1. The endophytic PGPR TNSC2011 can utilize indole acetic acid, as well as insoluble phosphate as a phosphorus source to support its growth.

Escherichia coli strain DH5α carrying the pgfp 4412 plasmid was grown in LB medium supplemented with ampicillin (100 μg/ml) at 37°C. The plasmid, pgfp4412, containing one copy of constitutively expressed gfp and neomycin- and ampicillin-resistance genes in tandem, was donated by the College of Agronomy and Biotechnology, China Agricultura University, Beijing, China (Tian *et al*., 2004).

Plasmid DNA extraction and transformation of endophytic bacteria

Escherichia coli DH5α was grown overnight in LB medium supplemented with ampicillin (100 μg/ml), and the plasmid was extracted using the Plasmid Midi kit (TIANGEN Biotech [Beijing] Co., Ltd.).

Electrocompetent cells of *A. marplatensis* strain 17 were obtained as follows: an overnight culture was diluted in a new fresh medium (1:50), and its growth was monitored until the cells reached OD_{600}=0.4–0.7. Cells were then harvested by centrifugation (10,000 × *g*, 10 min, 4°C) and washed three times with ice-cold sterile purified water. The cell suspension was concentrated 50-fold in 10% sterile glycerol and kept at -80°C.

Electrocompetent cells were transformed by electroporation (2.5 kv, 25 μf, 200 Ω, 4.5 ms) with plasmid pgfp 4412. Transformed bacteria were cultured on LB agar medium supplemented with ampicillin (100 μg/ml).

Detection of *in vitro* plasmid stability and plasmid gene expression: The stability of plasmid pgfp4412 was monitored throughout 72 h of cultivation of the transformed isolate. During the cultivation, 10 μl of culture was inoculated into new tubes every 24 h. For every inoculation, aliquots were plated on LB medium and 100 random single colonies were transferred using a toothpick to LB agar medium supplemented with 100 μg/ml of ampicillin. Colonies were counted, and the percentage of clones carrying the plasmid was estimated. All experiments were repeated twice.

Heterologous gene expression was determined after 24 h of growth on plates containing the LB solid medium supplemented with ampicillin (100 μg/ml). Bacteria grown in the presence of antibiotics and expressing the *gfp* gene, detected under UV light at 300–360 nm, were confirmed as carrying both features.

Growth kinetics of labeled strains

17 and 17:gfp strains were crossed following their individual culture and a single colony was selected and inoculated into LB liquid medium (the latter requiring the addition of kanamycin) and cultured overnight. Five milliliter of LB containing the bacteria was washed three times to remove the antibiotics and bacterial metabolites, and then the bacteria were resuspended in 5 ml of LB to carry out liquid colorimetric determination of the bacterial density at A600 nm. The suspension with sterile water regulation of bacteria, two strains of the A600 nm value, then the two strains of bacterial suspension washed by 1% respectively. (V/V) the inoculation amount to antibiotic free LB medium in a 250-ml flask. 100 ml of medium, 200 r/min in reciprocating thermostatic oscillator at 30°C, training, each treatment was repeated three

times. At a set time, samples of bacteria were taken and bacterial turbidity determined to enable a growth curve to be plotted.

Plant inoculation and enumeration of culturable endophytes in plant tissues: Sugar beet plants were propagated using seeds obtained from a commercial variety supplied by a local company, with surface sterilization. Before inoculation, roots of seedlings (21 days postgermination) were washed in water, soaked in a bacterial suspension [10^9 colony-forming units (CFU)/ml], and suspended in phosphate-buffered saline [PBS, containing (g/L) Na_2HPO_4 1.44; KH_2PO_4 0.24; KCl 0.20; NaCl 8.00; pH 7.4] for 1 h. Plants were either non-inoculated (control) or inoculated with *A. marplatensis* strain TNSC2011, as detailed above. Plants were then transplanted into individual pots (one seedling per pot) containing non-sterilized commercial substrate compost (turf, expanded vermiculite, and composted material of pine bark) and were maintained in a greenhouse with a 14-h photoperiod and 22/28°C day/night cycle for collection after 10 or 20 days. The experiment was performed with a complete randomized complete block design with 14 replicates for each treatment.

Plants were harvested, placed on a paper towel, and immediately transported to the laboratory, where the substrate was carefully removed from the roots. Stems and roots were separated and weighed. Surface disinfection was achieved by washing the plants with 70% ethanol for 1 min, with sodium hypochlorite solution (2% available Cl^-) for 2 min, and with 70% ethanol for 30 sec, followed by two rinses in sterile distilled water. To confirm that the endophytic bacteria reflected only the number of cells inside the plant tissues, a control procedure was always performed to ensure that proper surface sterilization occurred, in which aliquots of the sterile distilled water used in the final rinse were also plated on tryptic soy agar (TSA) medium amended with 100 µg/ml of benomyl to inhibit fungal growth. The plates were examined for growth after incubation at 28°C for 2–15 days. In a further step, pieces of stems and roots were cut into tiny fragments with the aid of a sterilized razor blade in sterile phosphate-buffered saline (1 ml for stems and 2 ml for roots) and serial dilutions were plated onto TSA as described above. Samples were also grown on TSA medium supplemented with ampicillin (100 µg/ml) to estimate the number of bacteria expressing *gfp*. The plates were incubated at 28°C for 2–15 days, after which the number of CFU was determined and the bacterial density was estimated.

In planta visualization of gfp-expressing endophytes using fluorescence microscopy

We selected the 17:gfp strain for the assessment of the colonization pattern based on its high-level green fluorescence intensity and persistence in periwinkle plants. Seedlings inoculated with 17:gfp were collected and examined using a fluorescence microscope at various times (24, 48, and 72 h). They were washed in running tap water, and placed separately on Petri dishes. Hand-cut sections of live leaves, stems, and roots were examined using an Axiophot-2 microscope (Zeiss), and images were captured with a charge-coupled device (CCD) camera, using the software Isis and Ikaros. The filter set 09 (Zeiss) with a 450–490 nm band-pass excitation and 550-nm emission was used for gfp examination. Phase-contrast microscopy was used to visualize the tissues, and the digital images were merged and processed.

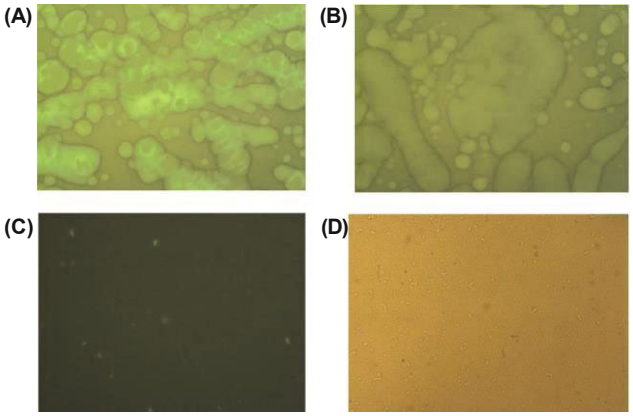

Fig. 1. Fluorescence of strain 17:gfp in culture solution (×1,000). The wild endophytic bacterium *Achromobacter marplatensis* 17 tagged with gfp (17:gfp) was observed under fluorescence microscopy. Bacterial colonies of bacterial cells with green fluorescence were displayed as bright green rods on a glass microscope slide.

Statistical analysis

All data were processed with Excel 2003 (Microsoft). Each data point in the figures and tables represents an average value. The standard deviation in parallel samples is shown in the figures as an error bar. Data were analyzed by ANOVA using SPSS version 11.0 (SPSS, Inc.) with a confidence limit of 95%.

Bacterial counts were transformed using $\log_{10}$ of x + 1 before the ANOVA. Tukey's test was used for comparison of means.

Results

Transformation of endophytic bacteria and *in vitro* plasmid stability

Transformation of *A. marplatensis* strain 17 with plasmid pgfp4412 resulted in nine transformants 2 days after transformation, of which one colony showed a high fluorescence. Its identity was confirmed in a TaqMan assay and by growth

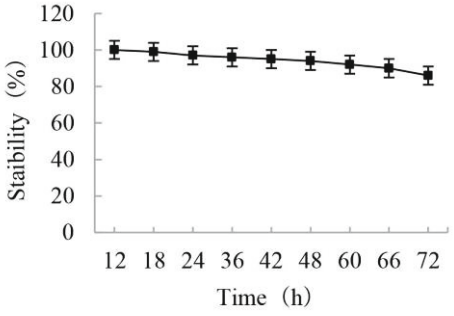

Fig. 2. Stability of engineered strain 17:gfp.

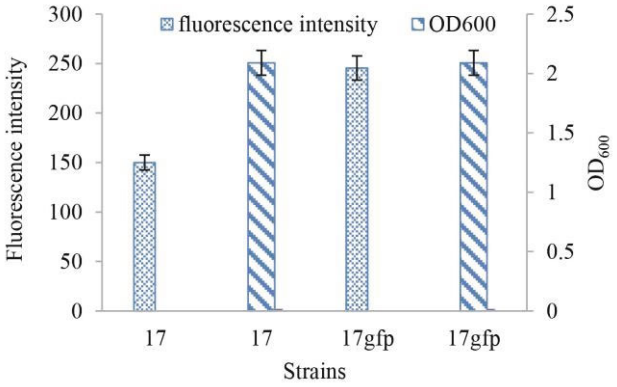

Fig. 3. **The fluorescence intensity and optical density of *Achromobacter marplatensis* 17 and *Achromobacter marplatensis* 17:gfp.**

on LB, on which colonies exhibited a dark color and a texture typical of *A. marplatensis* strain 17 (Fig. 1). Bacterial colonies of bacterial cells with green fluorescence were displayed as bright green rods on a glass microscope slide (Fig. 1A and B). The wild endophytic bacterium *Achromobacter marplatensis* 17 tagged with gfp (17:gfp) was observed under fluorescence microscopy (Fig. 1C and D). The presence of the plasmid was confirmed by plasmid DNA purification and agarose gel electrophoresis. The transformed plasmid was extracted and digested with enzyme, and then verified by electrophoresis.

The stability of the transformant under nonselective conditions was tested *in vitro* by transferring colonies to fresh LB medium without kanamycin every 2 days. The percentage of fluorescent colonies decreased gradually, but after 3 days, 86.0% of the colonies of strain 17 still fluoresced (Fig. 2). The transformant had a relative fluorescence intensity (FI) of 245.524 compared with an intensity of 150.099 FI for the parental strain (Fig. 3). The presence of the plasmid or the expression of the gfp protein did not influence the growth rate *in vitro* and growth of the transformant was similar to the parental wild-type strain.

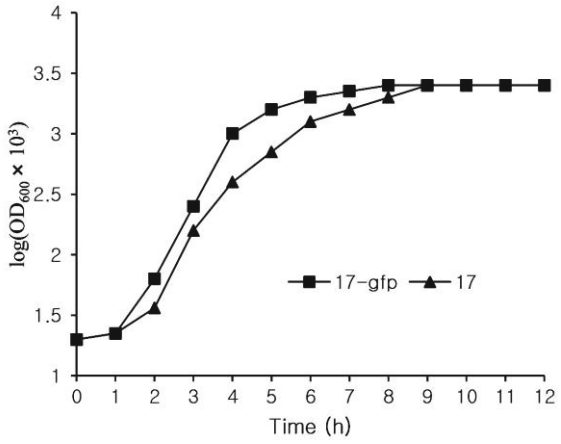

Fig. 4. **Growth curve of *Achromobacter marplatensis* 17 and *Achromobacter marplatensis* 17:gfp. The data are indicated as log ($OD_{600} \times 10^3$).**

When the plasmid pgfp4412 was introduced into *A. marplatensis* strain 17, up to 2×10^4 transformants/μg of plasmid DNA were obtained, indicating a high efficiency of transformation. A moderate efficiency of transformation (up to 4×10^3 transformants/μg of plasmid) was observed with the strain *E. cloacae* DH5α. The results showed that, after at least 24 h, most cells (97.0%) for 17:gfp were still carrying their respective plasmid. Moreover, after 72 h, the plasmid was detected in 86.0% of the cells of 17:gfp (Fig. 2).

Determination of growth kinetics curve

The growth kinetics curves of the wild-type strain 17 and the 17:gfp strain in the LB liquid medium are shown in Fig. 4. The growth curves of the strains were very similar. From the cell growth curve of the logarithm of the, as can be seen in the inoculum culture conditions, two strains were almost at the same time into the index growth period. The exponential growth phase of the wild-type strain ended 5 h after cultivation, whereas that of the 17:gfp strain ended 6 h after cultivation. According to the growth curve, the wild-type 17 and the marker strain 17:gfp in the LB liquid medium were calculated to be 0.99 h and 1.15 h, respectively, and the strain was extended by 6.7%.

Enumeration of cultivable endophytic populations

The reisolation of the endophytic bacteria from stems and roots was performed 10 and 20 days after inoculation. Total bacterial population and *gfp*-derived populations were determined for each of the tissues examined. The total population of endophytic bacteria in beet plants was estimated to be 10^5–10^6 CFU/g on medium without antibiotic. The endophytic population expressing *gfp* decreased slightly after 10 days in plants inoculated with 17:gfp under selective conditions (Fig. 5). In addition, we did not observe endophytes naturally resistant to ampicillin recovered from plants inoculated with the 17 strain. However, few morphological types were observed among the isolated endophytic bacteria in all treatments. The marker strain 17:gfp promotes plant growth similar to the wild-type 17. The plant height, fresh weight, dry weight and leaf number in seeds inoculated with the strain 17:gfp were increased by 56.1%, 58.4%, 98.7%, 212.2%, and 51.5%, respectively.

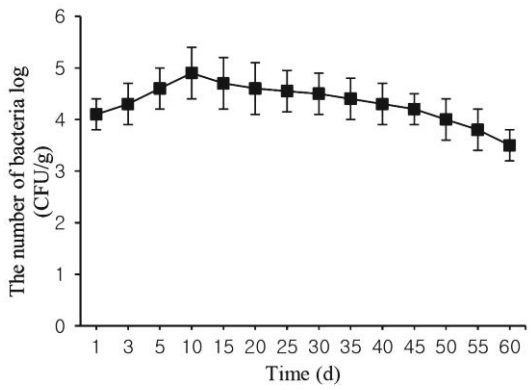

Fig. 5. **Colonization of *Achromobacter marplatensis* 17:gfp in sugar beet.**

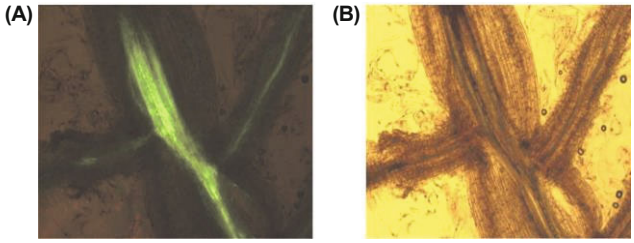

Fig. 6. Visualization of inoculated endophyte 17:gfp within plant tissues. After inoculation with 17:gfp for 10 days, the colonization of 17:gfp was observed in plant roots by fluorescence microscopy. Bacterial cells with green fluorescence were displayed as bright green rods inside roots (A). Plants with 17:gfp inoculation under visable light served as the control (B).

In planta visualization of gfp-tagged endophytes

Gfp-Expressing cells were visible at high densities in roots, and root hairs were clearly colonized (Fig. 6A). Stem cross-sections showing colonization by gfp-tagged cells were also observed 48 h after inoculation.

Few cells were observed in leaf tissues 24 h after inoculation and, although colonization increased 48 h after inoculation, with several cells being detected in the leaves, it decreased drastically 72 h after inoculation (data not shown).

Discussion

The plasmid pgfp4412 was stably maintained in the 17 strain for at least 72 h of bacterial cultivation on medium without antibiotics. The abundance of gfp-tagged bacteria colonizing the plant tissues did not suggest significant marker loss, highlighting the role of pgfp4412 as a valuable tool for studies of bacterial communities grown in the absence of antibiotic selection. The *gfp*-expressing strain was reisolated from the inner tissues of surface-sterilized roots and stems of inoculated plants, and the survival of *A. marplatensis* 17:gfp in plants 20 days after inoculation, even in the absence of selective pressure, suggests that this bacterium is a good colonizer. This observation is in agreement with results from a previous study (Verma *et al.*, 2004). The authors compared the endophytic colonization ability of two deep-water rice endophytes, *Pantoea* sp. and *Ochrobactrum* sp., using GFP. Although both *Pantoea* sp. and *Ochrobactrum* sp. were able to establish endophytically in the rice root, *Pantoea* sp. was the more aggressive invader in the rice tissues.

In this study, we used several strains of *E. coli* in the expression of the gfp shuttle vector pgfp4412 by electroporation of biocontrol *Achromobacter marplatensis* 17, and expression of *gfp*. The results showed that the *A. marplatensis* conversion efficiency was relatively low. However, this could be improved if the transformation process was more strictly controlled, including the transformation temperature and cleanliness of the vessels used. Adding a set concentration of state during the preparation of the cell HEPES could help to improve conversion efficiency, as can the addition of glucose (Verma *et al.*, 2004).

When using fungi to control soil-borne diseases, biocontrol bacteria in plant roots and the rhizosphere can have an effective role as biocontrol agents. Therefore, understanding the ability of biocontrol bacteria to colonize biocontrol fungi is an important part of the biocontrol mechanism, and has been widely reported (Haas and Défago, 2005).

Compared with other methods, the gfp marker has little influence on the strain, is easy to manipulate, and can be directly observed and evaluated. The use of plasmid pgfp4412, built with a cryptic plasmid pAD4412, probably favored the greatest expression of the *gfp* gene in that strain. Furthermore, the rapid spread of gfp-derived strains in sugar beet plants indicates that the vascular system is the probable route for systemic colonization, which is consistent with previous reports in other plants (Dong *et al.*, 2003; Germaine *et al.*, 2004; Chi *et al.*, 2005). It has also been suggested that *P. agglomerans* can be transported through the colonization of intercellular spaces in root and aerial tissues (Compant *et al.*, 2005). Populations of cultivable endophytes in both roots and stems of periwinkle plants were on average 10^4 CFU/g fresh weight, and similar values were previously reported in other plants (Kuklinsky-Sobral *et al.*, 2005; Andreote *et al.*, 2006; Lacava *et al.*, 2007). In addition, the cultivable indigenous endophytic population was markedly higher in the roots compared with the stems, which could result from the higher abundance of nutrients in the rhizosphere that can support bacterial growth and metabolism (Glick, 1995).

Another important feature is that the results from the current study indicate that *A. marplatensis* 17:gfp strain could be used as a vector to deliver enzymes or other proteins inside the plants, because their monitoring can be easily performed by detecting the expression of the *gfp* gene. Thus, it is unsurprising that *A. marplatensis* has been considered one of the most widespread groups in terms of its endophytic interactions with plants (Mareque *et al.*, 2015). This species has been found in studies using several plant species, such as citrus (Araújo *et al.*, 2001), eucalyptus (Ferreira *et al.*, 2008), and rice (Verma *et al.*, 2004).

In a recent study, the strain *A. marplatensis* 17:pgfp was able to colonize sugarcane plants, promoting their growth. The growth promotion observed in colonized plants could be related to the ability of *A. marplatensis* 17 to synthesize IAA and solubilize phosphate (Shi *et al.*, 2014). Furthermore, genetic engineering is easier in endophytic bacteria than in plants, and only one bacterium can successfully colonize multiple plants (Newman and Reynolds, 2005).

In summary, the gfp-tagged endophyte strain used in the current study was stable for at least 72 generations as well as after colonization of the plant tissues under nonselective conditions. Moreover, no damage was observed in inoculated plants, suggesting that these gfp-tagged strains, especially *A. marplatensis* 17, would be useful vehicles to use to deliver enzymes or other proteins *in planta*.

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Biological Control and Micro Ecology Laboratory of the China Agricultural University.

Conflict of Interest

We declare that we have no conflict of interest.

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