The colonization of Bacillus thuringiensis strains in bryophytes

Qiuqiu LIN1,2, Pengli ZHU1,2, Rebeca CARBALLAR-LEJARAZÚ3, Ivan GELBIČ4*, Xiong GUAN1,2, Lei XU1,2, Lingling ZHANG1,2*

1Key Laboratory of Biopesticide and Chemical Biology, Ministry of Education, Fujian Agriculture and Forestry University, Fuzhou, Fujian, P. R. China
2Fujian-Taiwan Joint Center for Ecological Control of Crop Pests, Fujian Agriculture and Forestry University, Fuzhou, P. R. China
3Department of Molecular Biology and Biochemistry, University of California Irvine, Irvine, CA, USA
4Biology Centre of the Czech Academy of Sciences, Institute of Entomology, České Budějovice, Czech Republic

Abstract: In our previous study, several Bacillus thuringiensis (Bt) strains were isolated from bryophyte samples, indicating that bryophytes could serve as Bt reservoirs in the wild. SFR13 is a wild strain isolated from the bryophyta Physcomitrium japonicum. In order to understand its ecological properties, green fluorescent protein (GFP)-labelled SFR13 (SFR13GFP) was generated to evaluate the colonization capability in bryophytes, using dynamic tracing and cell counting to observe the process and patterns of colonization. Our results showed that genetic stability, growth curve dynamics, and insecticidal crystal production were not affected by GFP expression in Bt. Fluorescence microscopy was used to track the dynamic distribution of SFR13GFP. Distribution patterns showed that SFR13GFP can establish stable and long-term colonization in leaves and stems by the 26th day after inoculation. A better understanding of how Bt colonizes plants in the wild will not only result in increased knowledge of plant–microbe interactions but will also lead to a more successful and reliable use of bacterial inoculants.

Key words: Bacillus thuringiensis, GFP, plant colonization, interaction mechanism, microscopy

1. Introduction

The use of environmentally safe insecticides based on Bacillus thuringiensis toxins (Bt, ubiquitous gram-positive, spore-forming soil bacterium) as an effective insect control strategy is well documented (Kumar et al., 2008; Vasquez et al., 2009). Furthermore, Bt arises from natural habitats including soil (Martin and Travers, 1989; Hastowo et al., 2014), insects (Carozzi et al., 1991; Cavados et al., 2014), stored products (Meadows et al., 1992), aquatic environments (Ichimatsu et al., 2000), feces (Lee et al., 2003; Ohba and Lee, 2003), and plants (Ohba, 1996; Mizuki et al., 1999; Zhang et al., 2007; Maduell et al., 2008; Zhang et al., 2010).

The commercial application of Bt requires both efficient colonization of plant tissues and the long-term maintenance of insecticidal activity inside the plant. Recently, the tracking of bacterial strains in plants has been reported that can be done with the use of fluorescently labelled bacteria, together with quantitative techniques (i.e. the plate counting method) and fluorescence microscopy (Krzyzanowska et al., 2012). Bt spores can subsist for a long time after spray applications; however, spore count methods for bacteria detection are limited (Schnepf et al., 1998). Although there are several studies about Bt colonization in plants (Damgaard, 1998; Bizzarri and Bishop, 2008; Ohba, 2011), it is still largely unclear how Bt can colonize plants or exert its pathogenic properties in soil in nature.

Bryophytes are among the simplest terrestrial plants, and are the most ancient lineage of terrestrial plants, dating to the early Ordovician period (488 million to 444 million years ago), and their study can provide key knowledge on early terrestrial diversification (Renzaglia et al., 2007). Most representatives lack complex tissue organization, yet they show considerable diversity in form and ecology (Ah-Peng et al., 2007). Moreover, they are widely distributed throughout the world and are relatively small compared with most seed-bearing plants. Bryophytes exhibit generational alternation, characterized by independent gametophyte generation, which produces the sex organs, sperm, and eggs, and dependent sporophyte generation, which produces the spores (Proctor and Tuba, 2002; Meyer et al., 2008).

In bryophytes, the composing parts include the stem, leaf, and rhizoid. When mature the gametophyte shows differentiation into stem and leaves but there are no roots.

* Correspondence: gelbic@entu.cas.cz; lingling00264@163.com
Compared with the stem and rhizoid, the leaf, without ribs, consists of a simple epithelium in which there are some chloroplast cells. The stem can be divided into two parts: big cells cortex and small cells axis stalk. The cortical tissue of the leafy stem consists of elongate parenchyma cells (150–250 mm) (Ligrone et al., 2000).

These simple plants are very important in initiating soil formation on barren terrain, in maintaining soil moisture, and in recycling nutrients in forest vegetation. Indeed, discerning the presence of particular bryophytes is useful in assessing the productivity and nutrient status of forest types (Bond-Lamberty et al., 2011; Cutler, 2011). Further, through the study of bryophytes, various biological phenomena have been discovered that have had a profound influence on the development of research in areas such as genetics and cytology. With multiple varieties, huge amount, simple structure, and extensive distribution, bryophytes also have obvious advantages as sources of Bt strains.

However, compared with chemical pesticides, there are some disadvantages of Bt biological agents, such as narrow spectrum and low toxicity, which lead to its high cost and greatly limit further application. Therefore, improving the ability of colonization of Bt is a useful way to enrich the resistance mechanism of plants against insects. However, the mechanisms of colonization, migration, and propagation of Bt in bryophytes are still poorly understood.

In our previous study, several Bt strains were isolated from 76 bryophyte samples, indicating that bryophytes can serve as Bt reservoirs in the wild (Zhang et al., 2007). The basic aim of the present study was to evaluate the colonization capability of Bt gfp-labelled strains in bryophytes, via dynamic tracing and cell counting, to further clarify and define the ecological significance of Bt, thus enabling the development of new strategies for biocontrol of plant pests and an improved theoretical and applied knowledge base of Bt ecological characteristics.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Bt isolate SFR13 was grown in Luria-Bertani (LB) medium at 30 °C. The gfp-tagged strain transformed with pCM20 plasmid was grown in LB medium with 100 µg/mL erythromycin at 30 °C.

2.2. Plasmids and bacterial transformation

The plasmid pCM20 was used to express the gfp gene in the Bt SFR13 strain and carries the 750 bp gfp and erythromycin resistance genes. The vector was introduced into Bt cells by electroporation (2.5 kV, 25 µF, 200 Ω) using a Bio-Rad Gene Pulser. Five milliliters of LB was inoculated with a single colony of Bt SFR13 and cultivated overnight at 30 °C and 150 rpm until OD600 of 0.9. Then SFR13 was chilled on ice for 30 min, and harvested by centrifugation at 6000 × g for 15 min at 4 °C after being washed four times with cold sterile distilled water. Next 100 µL of electro-competent cells were transformed with 3 µg of pCM20 DNA. Transformant bacterium (SFR13GFP) was mixed with 1800 µL of LB containing erythromycin (100 µg/mL), and grown at 30 °C for 3 h, and then plated on LB agar with Em at 30 °C until single colonies growth was observed.

2.3. Expression of GFP in Bt transformants

A comparison between SFR13 and SFR13GFP growth rates was evaluated by which a single colony of these two strains was cultured respectively overnight at 30 °C, followed by transferring into fresh LB by 1:100 dilutions. Two milliliters of each cell suspension was used to determine Bt growth by optical density at OD600 using a UV-Vis spectrophotometer (Cary 50 probe Varian) at 2-h intervals until sporulation. Aliquots of the cultures were observed by microscopy (magnification 100× with immersion oil) every 6 h to determine whether or not spores had been completely released.

Plasmid pCM20 stability was measured over 80 h from the beginning of transformant cultivation. A single colony of the SFR13GFP strain was cultured in LB medium supplemented with erythromycin (100 µg/mL). During the culture period, 1% of the bacterial suspension was used to inoculate fresh LB cultures (30 °C with 150 rpm), without antibiotic, every 12 h. After the first, third, fifth, seventh, and ninth inoculations, the subcultures were plated on LB agar and random single colonies were selected for fluorescence microscopy analysis and the percentage of clones carrying the pCM20GFP plasmid, under nonselection pressure, was calculated according to the method described by Fan et al. (2012). The expression level of GFP in Bt bacteria was determined as a function of fluorescence intensity (Kim et al., 2007).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is usually used to analyze relative molecular mass, the variety of major proteins, and the distribution of proteins of SFR13 and SFR13GFP. Based on the method described by Kaelin and Gadani (2000), the electrophoresis was prepared with 5% stacking (80 V) and 10% separating gels (100 V) with steady voltage. After staining with 0.04% Coomassie brilliant blue (R-250), the molecular masses of the proteins were detected with protein standards (Sigma).

2.4. Bt inoculation with SFR13GFP

The Bt strain SFR13GFP was grown in LB, plus erythromycin (100 µg/mL) at 30 °C until spores were completely released, and used in subsequent inoculation. Funaria hygrometrica plants (~200) from the herb garden of Fujian Agriculture and Forestry University were inoculated by soaking the leaves in 10 mL of diluted overnight culture of SFR13GFP (10⁶ spores cells mL⁻¹); as
control, the same number of equivalent plants were treated with sterilized water.

2.5. Microscopic observations
Twenty samples of 2-cm dimensional measurement leaves and stems were selected randomly from treated and control plant groups at 1, 3, 6, 10, 15, 20, and 26 days after Bt inoculation using a fluorescence microscope (Leica IX71, Japan). Green fluorescence emitted from plant tissue samples was observed using 488 nm excitation and 500–550 nm emission wavelength filter settings.

2.6. Quantification of SFR13GFP in bryophyte tissues
After 1, 3, 6, 10, 15, 20 and 26 days postinoculation, approximately 0.02 g of bryophyte was taken to the laboratory and used to quantify the Bt level in plant tissues. The tissues were separated into leaves and stems, then washed in 1 mL of sterilized water for 10 min, then blotted surface dry, and weighed. Thus Bt on tissues surface was obtained in 1 mL of water. Each individual preparation of 1 mL of Bt water divided into 2 aliquots: aliquot 1 was grown in LB containing erythromycin (100 µg/mL) to quantify the total number of Bt on the tissues surface and aliquot 2 was treated at 80 °C for 10 min to kill vegetative cells and grown in LB to quantify the number of spores on the tissues surface. To quantify the number of Bt that colonized in the inner of plant tissues, all samples were sterilized in 70% ethanol for 30 s and 0.1% mercuric chloride for 2 min and washed with sterilized water three times to remove the surface remaining bacteria, according to the method described by Fan et al. (2012). The last water-rinse solution was plated in LB-agar plates and used to determine the efficiency of surface sterilization and confirm that the bacteria reflected only the number of cells inside the plant tissues. The surface sterilized plant tissues (stems and leaves) were homogenized with 1 mL of sterile water using a mortar and pestle. Then each individual preparation of grinding fluid with Bt was divided into 2 aliquots and processed as mentioned earlier to quantify the number of Bt within the inner tissues, such total number, spores, and vegetative cells of Bt in leaves and stems.

All plant extracts were subject to serial dilutions (1:10, 1:100, and 1:1000) and plated on LB-agar medium containing erythromycin (100 µg/mL) and incubated at 30 °C for 18 h. Observed bacteria colonies were assayed for the presence or absence of the green fluorescence phenotype using a fluorescence microscope (Leica IX71, Japan) with 488 nm excitation and 500–550 nm emission wavelength filter settings.

3. Results and discussion
3.1. GFP expression in Bt SFR13GFP strain
A common strategy to study plant–microbe interactions is the use of fluorescent proteins, such as the GFP (Gau et al., 2002; Maduell et al., 2008). In the present study, Bt strain SFR13 was successfully transformed with the plasmid pCM20 to express GFP. Transformants were evaluated by fluorescent microscopy (Figure 1) and RT-PCR (data not shown), and showed bright fluorescence that was easily detected and the intensity of the purified GFP-displaying spores was uniform. However, GFP fluorescence reported variable expression levels between individual single cells, most probably explained by differences in transformation efficiency (Figure 1). Importantly, the transformants did not differ from the wild-type strain in either morphology or stability.

3.2. Biological characteristics of SFR13GFP tagged strain
A comparison between the SFR13GFP and the parental wild-type strains showed that they exhibited similar growth curves in both the logarithmic and stable phases. Yet, the

Figure 1. GFP expression in Bt recombinant strain SFR13GFP. Fluorescence microscopy (FM) was used to detect GFP expression in Bt colonies transformed with pCM20 plasmid. (A) SFR13GFP colonies at 4× magnification, (B) SFR13GFP cells at 40× magnification.
wild-type strain reached the logarithmic growth phase a little earlier than SFR13GFP and had higher maximum cells at the stationary phase. It could be deduced that GFP expression had little effect on the stability and growth of Bt; there is no significant difference in Bt growth (Figure 2). During the first 12 h of culture (after one transfer) no loss of plasmid was observed, while after 36 h 80% of cells were still GFP positive, indicating that GFP can be efficiently and stably expressed up to 36 h in culture without any selective pressure. We also observed the same crystal protein pattern in SRF13GFP as in the wild-type strain, indicating that GFP expression does not alter the crystal composition in Bt (Figure 3). Our results showed that Bt SFR13 gfp-tagging is possible, as has been reported for Paenibacillus polymyxa isolate B1 (Timmusk et al., 2005), and provides an important tool to further study Bt colonization in bryophytes.

3.3. Recovery of SFR13GFP isolates from different plant tissues

The wild-type strain SFR13 was used as a model for bryophyte colonization in the present study. Lodewyckx et al. (2002) previously described the potential of bacteria to colonize endophytes roots, stems, and leaves, tissues that are relatively protected from the competitive and high-stress environment of the soil (Kobayashi, 2000). These results show that Bt can colonize bryophyte tissues and that the colonization can still be observed after a month.

The population dynamics of Bt SFR13GFP and its efficiency in bryophyte colonization were determined via the reisolation of the gfp-tagged bacterial population from

![Figure 2](image2.png)

**Figure 2.** Growth comparison between SFR13 and SFR13GFP strains. Relative values are based on biomass every 2 h during culture time. Each value and error bar represents the mean of three independent experiments and their standard deviation (t-test, P = 0.014).

![Figure 3](image3.png)

**Figure 3.** SDS-PAGE analysis from SFR13 (lane 1) and SFR13GFP (lane 2) crystal proteins. No effect in Bt crystals was observed after the transformation with pCM20 plasmid. M, Protein marker.
stems and leaves at different time points after the initial inoculation (1, 3, 6, 10, 15, 20, 26 days). We found that 24 h after *Bt* inoculation SFR13GFP density was up to $6.3 \times 10^6$ colony forming units per gram (cfu/g) in leaves and $2.6 \times 10^6$ cfu/g in stem surfaces. The life cycle of *Bt* is characterized by 2 phases, including vegetative cell division and spore development, otherwise referred to as the sporulation cycle. As the SFR13GFP density varied between the surfaces and interior of the tissue (Figures 4 and 5) and most of the tagged strain was observed in the vegetative form, we suppose that *Bt* SFR13GFP had colonized the plant tissue. We also observed that the bacteria population decreased to $10^5$ cfu/g outside the leaves 6, 10, and 15 days postinoculation and stayed over $10^6$ cfu/g within other times after inoculation outside stems and leaves. The bacteria population showed a certain degree of random variability in real data inside stems and leaves. *Bt* vegetative forms comprised 50% of the total population, indicating that the *Bt* gfp-tagged strain was able to massively colonize plant tissues (Figures 4 and 5).

We also recovered SFR13GFP from internal plant tissues. Among the 7 time points examined, GFP-tagged cells showed strong colonization ability with the bacteria population naturally beginning to multiply, being $2.0 \times 10^6$ cfu/g in leaves and $1.5 \times 10^6$ cfu/g in stems 3 days after inoculation. Vegetative *Bt* forms also increased in number compared to spores. This is important because vegetative cells of *Bt* can adhere and enter crop seeds after 45 days (Tanuja et al., 2013). However, in contrast to this report,
our observation of plant tissues at a high magnification indicated that fluorescent bacteria penetrate the intercellular space 3 days after inoculation and colonization took place as early as 10 days postinoculation. It could therefore be deduced that SFR13GFP may preferentially colonize bryophytes by an order of magnitude fluctuation.

3.4. SFR13GFP colonization model in bryophytes

Colonization in bryophyte tissues was observed after 10 days postinoculation. Fluorescence microscopy analysis was carried out on live stems and leaves, and SFR13GFP cells were clearly detected along the stems, especially in the interval. The number of detectable cells increased considerably 1 day after the initial spraying inoculation, and stayed at $10^6$ cfu/g steadily on the surface of stems. A large population existed inside the stems on these days except for 1 day after inoculation. GFP-tagged cells were also observed in tissues and on the surface of leaves. Interestingly, more Bt was detected in the patch regions of leaves, suggesting these may be effective entry sites for Bt infection. It was also observed that SFR13GFP cells seemed to adapt themselves to the surface shape of leaf tips (Figure 6).

In our study, about $10^6$ cfu/g total cells in each tissue could be recovered from bryophytes; the population sizes on stems and leaves were close to the expected concentration, in this initial range. The existence of spores and vegetative cells accounts for their different growth conditions in natural environments. The survival of the SFR13GFP in plants 26 days after inoculation demonstrates that they were able to colonize rapidly and efficiently even in the existence of pressure from outside. Fluorescence microscopy observations showed SFR13GFP cells often spread along the surfaces of stems and leaves and within the internal tissues of these structures, even under natural conditions. The research described here provides a foundation for the future investigation of Bt ecology, a field where a great deal remains to be uncovered.

A better understanding on how Bt colonizes plants in the wild will not only result in increased knowledge of plant–microbe interactions but will also lead to more successful and reliable use of bacterial inoculants. Our observations confirm that SFR13GFP can be used as an effective tool for subsequent colonization. Nevertheless, the mechanism of bacterial ingress into the internal tissues of bryophyte stems and leaves remains to be determined, and thus requires further investigation.

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Figure 6. Fluorescence microscope micrographs of bryophyte tissues colonized by SFR13GFP. Bryophytes were inoculated with SFR13GFP ($10^7$ CFU/mL) and tissues microscopically analyzed for GFP fluorescence. Images were visualized 10 days after inoculation by Leica IX71 fluorescence microscope. Green pseudocolor indicates GFP-expressing minicolonies. Panels A and B recorded the colonization of SFR13GFP cells on the leaves. Panels C and D recorded that GFP-tagged cells existed the intercellular spaces of the stem.
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