ORIGINAL PAPER

# Impact of transgenic Bt-cotton on the diversity of pink-pigmented facultative methylotrophs

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**Abstract** Pink-pigmented facultative methylotrophs (PPFMs) are one of the beneficial proteobacteria commonly found in phyllosphere, rhizosphere and as endophytes in cotton. To assess the impact of transgenic Bt-cotton on changes in the diversity and community profile of PPFMs by comparing with its non-transgenic cotton, a polyphasic approach including differential carbon-substrate utilization profiling and DNA fingerprinting techniques like ARDRA, RISA, BOX-PCR and ERIC-PCR were studied. PPFMs from phyllosphere, rhizoplane and internal tissues of the stem of both Bt-cotton and non-Bt-cotton were isolated and analysed in this study. All the results suggested that the diversity richness of PPFMs present in the phyllosphere, rhizoplane and internal tissues did not differ between Bt- and non-Bt-cotton. In this study, there was no evidence to indicate any adverse effects of Bt-cotton on the diversity of plant-associated methylobacteria.

**Keywords** Transgenic Bt-cotton · PPFM · *Methylobacterium* · Diversity · ARDRA · RISA · BOX-PCR · ERIC-PCR

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## Introduction

Transgenic plants display considerable potential to benefit both developed and developing countries. Expressing insecticidal proteins, proteins providing tolerance to herbicides and resistance to environmental stresses are some of the achievements made so far by transgenic plants (Shelton et al. 2002; Velkov et al. 2005). The use of transgenic crops for pest management has risen dramatically since their introduction in mid 1990s. The transgenic crops are an environmentally safe tool used to decrease the amount of chemical pesticides in agriculture. One of the most commonly used transgenic crops is Bt-cotton, which constitutively expresses the insecticidal toxin from *Bacillus thuringiensis* encoded by the truncated *cryIAC* gene. Concerns have been raised about the environmental risks associated with the release of transgenic crops, including the potential impact on non-target organisms such as beneficial insects, soil bacteria and fungi, which play a fundamental role in soil fertility (Dale et al. 2002).

Structural and functional diversities of microorganisms in the rhizosphere differ among the plant species due to differences in root exudates. Microbial communities associated with rhizospheres of transgenic cotton (*Gossypium hirsutum* L.) and alfalfa (*Medicago sativa*) significantly differed from these of the non-transgenic isogenic lines (DiGiovanni et al. 1999; Donegan et al. 1995). In contrast, *B. thuringiensis* toxins present in transgenic corn (*Zea mays* L.) rhizosphere did not change culturable microbial communities (Saxena and Stotsky 2001). The diversity of rhizosphere bacteria of transgenic, herbicide-resistant corn was not different from that of the corresponding non-transgenic variety (Schmalenberger and Tebbe 2002). A similar study conducted in the Bt-corn rhizosphere revealed no significant variation in the diversity of bacterial

community both in Bt-corn and non-Bt-corn (Brusetti et al. 2004; Fang et al. 2005). These studies illustrate the various impacts of transgenic crops on soil microorganisms.

Pink-pigmented facultative methylotrophs (PPFMs), soil proteobacteria, are commonly found in association with plants and have been hypothesized to potentially dominate the phyllosphere bacterial population (Corpe and Rheem 1989). They belong to the α2 subgroup of Proteobacteria, growing on wide range of one carbon compounds such as formate, formaldehyde, methanol, methylamine etc (Patt et al. 1976). The common PPFM, *Methylobacterium* sp., has been found in the phyllosphere of tropical crops, especially in cotton. The PPFMs offer the associative symbiotic life with crop plants by utilizing the methanol emitted through the leaves and in turn provide cytokinins to the plants (Lidstrom and Chistoserdova 2002). These bacteria are present in soil, phyllosphere, rhizosphere and as endophytes in cotton (Madhaiyan et al. 2006). The hypothesis of this study is that secretion of Bt-toxin through root exudates and accumulation in leaves and inner tissues of cotton may affect the colonization and community structure of PPFMs as non-target organisms, which in turn affects the plant–microbe interaction. To find out whether this is so, we studied the PPFM community structure in phyllosphere, rhizoplane and internal tissues of Bt-cotton with its counterpart non-Bt-cotton cultivar.

Many reports are now available demonstrating the utility of PCR for fingerprinting of many organisms including soil proteobacteria such as rhizobia (Olive and Bean 1999). A rapid, reliable and sensitive PCR method to differentiate the species is the amplified ribosomal DNA restriction analysis (ARDRA), which involves PCR amplification of the 16S rRNA gene using specific primers followed by digestion with one or more selected restriction enzymes (Heyndricxs et al. 1996). Although the 16S rRNA gene has been widely used for fingerprinting, the analysis of intergenic spacer region (IGS) between 16S and 23S rRNA gene (referred as Ribosomal Intergenic Spacer Analysis, RISA) has also been shown to be species-specific and has been successfully used to fingerprint simple communities (Ranjard et al. 2000). Enterobacterial repetitive intergenic consensus (ERIC) and BOX elements (BOX) like repetitive extragenic palindromic elements are described in a vast variety of eubacterial species and now ERIC- and BOX-PCR fingerprinting have been widely used for typing gram-positive and gram-negative organisms (Finger et al. 2006). Therefore, we used a polyphasic approach which included differential carbon-substrate utilization profile and ARDRA, RISA, ERIC- and BOX- PCR-like DNA-based molecular fingerprinting analysis to assess the impact of transgenic cotton on the PPFM community.

## Materials and methods

### Plant growth conditions and PPFM isolation

Bt-cotton (*Gossypium hirsutum*, cultivar NCS145 BunnyBt) and its non-Bt counterpart (cultivar NCS145 Bunny) were grown in the experimental plots of Tamil Nadu Agricultural University, Coimbatore, India and were used for this study. Both the cultivars were sown and maintained in a same field where the soil's physical, chemical and microbiological conditions were the same. Both of the crops did not receive any herbicides, pesticides and foliar sprays till the cropping. Ten plants of each cultivar were selected randomly at 45 and 90 days after sowing and the fifth and seventh leaves of the main stem, root samples and main stem portions (30 cm from the top) were collected in sterile polybags and isolation of PPFMs was performed as described earlier (Holland and Polacco 1994). Isolation and culturing of PPFMs were performed using ammonium mineral salt medium supplemented with 0.1% filter-sterilized methanol (MMS medium). Phyllosphere PPFMs were isolated by leaf impression technique on the same day as previously described (Holland and Polacco 1994). Root samples were washed several times with sterile water until free of soil particles, crushed with sterile 0.1 M sodium phosphate buffer (pH 7.0) and spread on the surface of MMS agar plates. Similarly, stem samples were surface sterilized with 70% alcohol for 1 min, washed several times with sterile water, cut into 0.5 cm bits, crushed with sterile 0.1 M sodium phosphate buffer (pH 7.0) and spread on the surface of MMS agar plates. Well-pigmented (pink-coloured) PPFM colonies grown on methanol medium were purified and maintained in the same medium at 30°C. The type cultures and standard strains of various plant-associated PPFMs used in this study are presented in Table 1. These cultures were also maintained in MMS medium at 30°C.

### Authentication of PPFM isolates

The presence of the *mxaF* gene in the isolates, encoding methanol dehydrogenase a key enzyme for PPFMs, was used for authentication of the isolates. Presence of the *mxaF* gene in the isolates was detected by partial amplification of the gene using specific primers (McDonald et al. 1995). A single actively grown PPFM colony was dispersed in 15 μl of sterile water in 1.5 ml eppendorf tubes and heated in a water bath at 95°C for 10 min. After cooling with ice, 5 μl of lysate was used as template DNA. PCR products were separated by electrophoresis on 1.5% agarose gels stained with ethidium bromide and documented in the Alpha Imager™1200 documentation and analysis system.

**Table 1** Details of standard/type strains of *Methylobacterium* species used in this study

| *Methylobacterium* species | Strain/type strain | 16S rRNA gene GenBank accession number | Source |
|---|---|---|---|
| *M. aminovorans* | TNAU9 | EF116587 | Present study |
| *M. extorquens* | DSM1337[T] | AB175633 | T. Sa, Korea |
| *M. fujisawaense* | DSM5686[T] | AJ250801 | T. Sa, Korea |
| *M. mesophilicum* | DSM1708[T] | AB175636 | T. Sa, Korea |
| *M. nodulans* | ORS2060[T] | AF220763 | P. Jourand, France |
| *M. populi* | TNAU12 | EF116590 | Present study |
| *M. radiotolerans* | DSM1819[T] | AB175640 | T. Sa, Korea |
| *M. suomiense* | TNAU4 | EF116584 | Present study |
| *M.* sp. | TNAU14 | EF116592 | Present study |
| *M. thiocyanatum* | TNAU8 | EF116586 | Present study |

### Carbon utilization test

All the PPFM isolates and standard strains of *Methylobacterium* species were analysed for growth on different carbon compounds viz., acetate, betaine, citrate, fructose, D-glucose, L-glutamate, methylamine, D-xylose, thiocyanate, tartarate, arabinose, dimethyamine, trimethylamine and cyanate. These carbon compounds were substituted for methanol in AMS liquid medium at 0.5% (w/v) level. Presence of growth was observed after 15 days of incubation at 30°C in an orbital shaking incubator and growth was compared to a negative control containing no added carbon source.

### Genomic DNA extraction

PPFM isolates were grown in AMS broth for 7 days and genomic DNA was extracted by the hexadecyltrimethylammonium bromide (CTAB) method as described by Ivanova et al. (2000). The integrity and concentration of purified DNA was determined by agarose gel electrophoresis (Sambrook and Russell 2000). The total genomic DNA extracted was dissolved in sterile distilled water and stored at 4°C. The concentration of total genomic DNA was adjusted to a final concentration of 20 ng/µl for PCR amplification.

### ARDRA and RISA

The 16S rRNA gene and the intergenic spacer (IGS) region between 16S and 23S rDNA were amplified by following the procedures of Sy et al. (2001) and Martin-Laurent et al. (2006), respectively. Both the PCR products were purified using PCR clean kit (Sigma GenElute™ PCR clean-up kit, USA) according to the manufacturer's instruction and approximately 1 µg of PCR amplified 16S rDNA and IGS were restricted with endonuclease *Hae*III (Fermentas, USA) separately at 37°C for overnight. The digestion mixture was separated by electrophoresis in 2.5% MetaPhor agarose gels.

### BOX- and ERIC-PCR

PCR was carried out with BOX and ERIC primers according to Versalovic et al. (1991) as modified of Hussain et al. (2004). The PCR conditions were carried out under similar conditions of Hussain et al. (2004) except that the extension temperature was 72°C for 2 min instead of 65°C for 8 min. PCR products were analysed by horizontal gel electrophoresis in 2.5% MetaPhor agarose gels.

### Similarity analysis

ARDRA, RISA, BOX-PCR and ERIC-PCR products resolved in 2.5% MetaPhor agarose gel electrophoresis were documented in the Alpha Imager™ 1200 documentation and analysis system. In order to determine the similarity between PPFM isolates, a binary matrix was established recording the presence or absence of bands in ARDRA, RISA, BOX-PCR and ERIC-PCR profiles. UPGMA algorithm was used for hierarchical cluster analysis. Pairwise comparisons were calculated using Jaccard's coefficient (Jaccard 1912) and a dendrogram was built using the UPGMA method (Nei and Li 1979) using the NTSYS-PC2 package.

### Results

The PPFMs were isolated from the phyllosphere, rhizoplane and inner tissues of the stem portion of Bt-cotton and its counterpart non-Bt-cotton cultivars. Among these three niches tested for occurrence of PPFMs, the phyllosphere of cotton harboured the maximum PPFM population followed by the rhizoplane. The inner tissues of cotton stems yielded very few endophytic PPFMs (Table 2). This difference of

**Table 2** Population of pink-pigmented methylotrophic bacteria in different regions of Bt- and non-Bt-cotton plant

| Niche | Population of PPFMs[c] | |
| --- | --- | --- |
| | Bt-cotton | Non-Bt-cotton |
| Phyllosphere[a] (c.f.u./cm$^2$ of leaf surface) | 497 ± 2.02 | 489 ± 6.63 |
| Rhizoplane[b] (c.f.u./g fresh weight of root) | 235 ± 3.70 | 244 ± 2.11 |
| Inner tissues of stem[b] (c.f.u./g fresh weight of tissue) | 38 ± 1.18 | 35 ± 1.92 |

[a] PPFM population was estimated by leaf-impression technique

[b] PPFM population was estimated by serial dilution-plating technique

[c] Data are mean ± standard error

PPFM occurrence in different parts the of cotton plant was showed the trend in Bt-cotton and non-Bt-cotton. All these PPFM isolates could grow well in MMS medium and were able to produce pink pigments after 3 days of incubation at 30°C.

Well grown, distinct pink-coloured PPFM isolates of 35 each from Bt-cotton and non-Bt-cotton representing all the three niches maintained in MMS agar were investigated for their metabolic and molecular diversity. Before the diversity analysis, all these isolates were authenticated for methylotrophy by partial amplification of the *mxaF* gene by rapid colony PCR. Except for one Bt-cotton isolate and two non-Bt-cotton isolates, all other PPFMs showed amplification of a partial *mxaF* gene about 550 bp in size. The authenticated isolates were subjected to differential carbon-substrate utilization profile, along with standard strains of methylobacterial species.

The isolates showing similar carbon-substrate utilization profile are grouped and presented along with standard strains of *Methylobacterium* species in Table 3. The majority of the PPFM isolates (both Bt- and non-Bt-cotton) coincided with the carbon-substrate utilization profile of already reported *Methylobacterium* sp. Most isolates had a carbon-substrate utilization profile similar to common plant-associated methylobacteria such as *M. suomiense*, *M. populi*, *M. thiocyanatum* and *M. aminovorans*. Six

**Table 3** Differential carbon-substrate utilization profile of the PPFM isolates from Bt-cotton and non-Bt-cotton and standard strains of *Methylobacterium*

| Carbon substrates[a] | Differential carbon-substrate utilizing PPFM isolate groups[b,c] | | | | | | Strains/type strains of *Methylobacterium* species | | | | | | | | | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| | I | II | III | IV | V | VI | Ma | Me | Mf | Mm | Mn | Mp | Mr | Ms | Msp | Mt |
| Methylamine | + | + | + | + | + | + | + | + | − | − | − | + | − | + | − | + |
| Acetate | + | + | + | + | + | + | + | + | + | − | + | + | + | + | + | + |
| Citrate | − | − | + | + | − | − | − | − | + | + | + | − | + | − | + | + |
| L-Glutamate | + | − | + | + | − | − | + | + | + | + | + | − | + | − | + | + |
| D-Glucose | − | + | + | − | + | − | − | − | + | + | − | − | + | + | + | + |
| D-xylose | − | − | + | − | − | − | − | − | + | − | + | − | + | − | + | + |
| Fructose | + | + | + | − | − | + | + | − | + | − | − | + | − | + | + | + |
| Betaine | + | + | + | − | − | + | + | + | − | − | + | + | + | + | − | + |
| Thiocyanate | − | − | + | + | − | − | − | − | − | − | − | − | − | − | − | + |
| Tartarate | − | − | − | − | − | + | − | + | + | − | + | + | − | + | + | + |
| Arabinose | + | − | − | − | − | − | + | − | + | + | + | − | − | − | + | + |
| Dimethyl amine | + | − | − | − | − | − | + | − | − | − | − | − | − | − | − | + |
| Trimethyl amine | + | − | − | − | − | − | + | − | − | − | − | − | − | − | − | − |
| Cyanate | − | − | + | + | − | − | − | − | − | − | − | − | − | − | − | − |
| Nutrient broth | + | + | + | + | + | + | + | + | + | − | + | + | + | + | + | + |

[a] The carbon substrates were substituted for methanol in MMS medium at 0.5% (w/v); +, growth; −, no growth

[b] All the PPFM isolates (both from Bt-cotton and non-Bt-cotton) were grouped based on similar carbon-substrate utilization profile

[c] Mean of three replicates

Ma—*M. aminovorans* (TNAU 9); Me—*M. extorquens* (DSM1337[T]); Mf—*M. fujisawaense* (DSM5686[T]); Mm—*M. mesophilicum* (DSM1708[T]); Mn—*M. nodulans* (ORS2060[T]); Mp—*M. populi* (TNAU12); Mr—*M. radiotolerans* (DSM1819[T]); Ms—*M. suomiense* (TNAU4); Msp—*Methylobacterium* sp. (TNAU14); Mt—*M. thiocyanatum* (TNAU8)

**Table 4** Distribution of PPFM isolates in phyllosphere, rhizoplane and internal tissues of Bt-cotton and non-Bt-cotton based on carbon-substrate utilization profile

| Differential carbon-substrate utilizing PPFM isolate groups | No. of PPFM isolates[a] | | | | | |
| --- | --- | --- | --- | --- | --- | --- |
| | Bt-cotton | | | Non-Bt-cotton | | |
| | Phyllosphere | Rhizoplane | Internal tissues | Phyllosphere | Rhizoplane | Internal tissues |
| I | 5 | 2 | 4 | 7 | – | 3 |
| II | 4 | – | – | 4 | – | – |
| III | 4 | – | – | 6 | – | – |
| IV | – | 4 | – | – | 4 | – |
| V | – | 4 | – | – | 4 | – |
| VI | 7 | – | – | – | 3 | – |

[a] Number of PPFM isolates showing similar carbon-substrates utilization profile in different niches of cotton plants is segregated

different groups of metabolically divergent PPFMs were reported among the isolates of Bt-cotton and non-Bt-cotton. Three metabolically diversified PPFM gropus were reported in the phyllosphere and rhizoplane of Bt-cotton and non-Bt-cotton, while the internal tissues harboured a single group of PPFM. However, the distribution of metabolically diversified PPFMs was not altered in Bt-cotton as compared to its counterpart non-Bt-cultivar (Table 4). From these six metabolically divergent PPFM isolates, seven isolates from Bt-cotton (isolates namely BBCL1, BBCL2, BBCL3, BBCR1, BBCR2, BBCR3, BBCS1) and non-Bt-cotton (isolates namely NBCL1, NBCL2, NBCL3, NBCR1, NBCR2, NBCR3, NBCS1) representing all the ecological niches and different carbon-substrate utilization profile groups were selected and analysed for molecular diversity to record the influence of transgenic crops. ARDRA, RISA, BOX- and ERIC-PCR fingerprinting techniques were employed to reveal the divergence of PPFMs between Bt-cotton and non-Bt-cotton.

Full length 16S rDNA and intergenic spacer (IGS) region between 16S and 23S rDNA of 14 PPFM isolates and 10 standard/type strains of *Methylobacterium* spp. were amplified by PCR and the products digested with *Hae*III enzyme. This enzyme was able to generate different restriction patterns among the isolates in both the 16S rDNA and IGS region amplicons. Figure 1 represents the restriction pattern of PPFM isolates of both Bt-cotton and non-Bt-cotton with methylobacterial standard strains, generated with ARDRA (Fig. 1a) and RISA (Fig. 1b). The resultant restriction pattern profile of ARDRA and RISA were analysed by UPGMA. Both methods generated similar results and the combined UPGMA cluster analysis is presented in Fig. 2.

In the UPGMA dendrogram, when applying 0.5 Jaccard's similarity coefficient for clustering, these restriction analyses grouped the PPFM isolates and standard strains of *Methylobacterium* into six different restriction types. Restriction pattern type I included the isolates BBCL1,



**Fig. 1** ARDRA (**a**) and RISA (**b**) profile of PPFM isolates from Bt-cotton and non-Bt-cotton and standard strains of *Methylobacterium* species. M—1 kb DNA marker ladder; Ms—*M. suomiense* (TNAU4); Mt—*M. thiocyanatum* (TNAU8); Ma—*M. aminovorans* (TNAU 9); Mp—*M. populi* (TNAU12); Mf—*M. fujisawaense* (DSM5686[T]); Mm—*M. mesophilicum* (DSM1708[T]); Me—*M. extorquens* (DSM1337[T]); Mr—*M. radiotolerans* (DSM1819[T]); Mn—*M. nodulans* (ORS2060[T]); Msp—*Methylobacterium* sp. (TNAU14). BBC—Bt-Bunny cotton; NBC—non-Bt-Bunny cotton
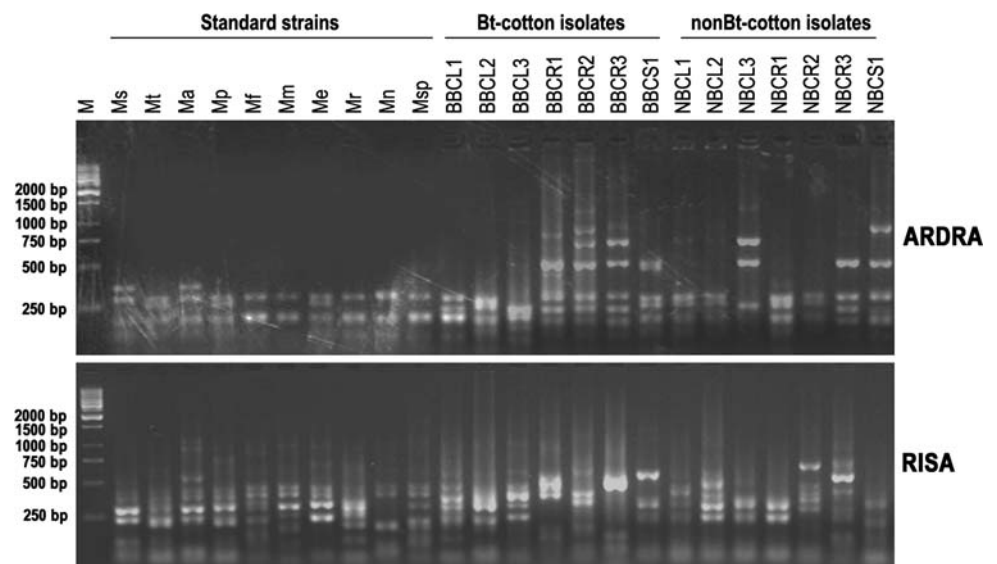
**Fig. 2** UPGMA dendrogram based on ARDRA and RISA profiles of PPFM isolates from Bt-cotton and non-Bt-cotton and standard strains of *Methylobacterium* species. The matrix generated with restriction pattern of 16S rRNA gene and ISR between 16S and 23S rDNA by *Hae*III enzyme. The coefficient used was Jaccard's similarity coefficient. BBC—Bt-Bunny cotton; NBC—non-Bt-Bunny cotton
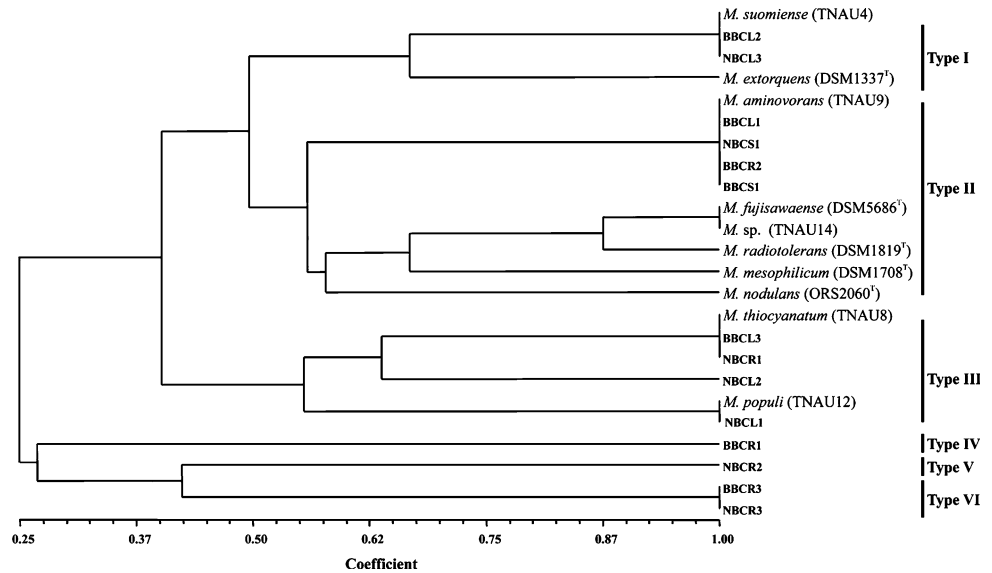
NBCL3 and strains of *M. suomiense* (TNAU4) and *M. extorquens* (DSM1337ᵀ). Type II included BBCL1, BBCR2, BBCS1, NBCS1 with *M. aminovorans* (TNAU9), *M. fujisawaense* (DSM5686ᵀ), *M. radiotolerans* (DSM1819ᵀ), *M. mesophilicum* (DSM1708ᵀ), *M. nodulans* (ORS2060ᵀ). Type III restriction pattern include BBCL3, NBCR1, NBCL1 and strains of *M. thiocyanatum* (TNAU8) and *M. populi* (TNAU12). Type IV and V had a single isolate BBCR1 and NBCR2, respectively, while type VI included BBCR3 and NBCR3 (Fig. 2).

Analysis of the fingerprint pattern obtained with BOX- and ERIC-PCR amplification also revealed a considerable genetic diversity among the PPFM isolates obtained from Bt-cotton and non-Bt-cotton. In Fig. 3, fingerprints of 14 PPFM isolates and 10 standard strains of *Methylobacterium* spp. generated with the BOX primer (Fig 3a) and ERIC primers (Fig 3b) are presented. The combined data analysed by Jaccard's similarity coefficient and developed the dendrogram by UPGMA is presented in Fig. 4. The reproducibility of each PCR genomic fingerprinting method was confirmed by repeating the amplification at least three times. Amplification of genomic DNA with BOX primer generated 8–14 DNA fragments for each isolate or strain tested and the size of the DNA band ranged from 0.1 to 1.5 kb (Fig. 3a). The amplification with ERIC primers generated more a complex amplification pattern than BOX-PCR (Fig. 3). Each strain generated 7–15 bands that ranged from 0.1 to 2.0 kb in ERIC-PCR. The fingerprints obtained with BOX- and ERIC-PCR of each strain were combined and a dendrogram was generated using Jaccard's similarity coefficient. The PPFM isolates and methylobacterial type strains formed six different clusters with more than 50% Jaccard's similarity index. These clusters are in accordance with the ARDRA and RISA

restriction pattern types with very slight variations (Fig. 4). However, ARDRA and RISA restriction pattern types as well as BOX- and ERIC-PCR fingerprint clusters revealed that the PPFMs' genetic diversity was not altered in Bt-cotton due to the transgenecity of the *cry* gene when compared to its counterpart non-Bt-cultivar.

## Discussion

There has been a strong debate on the safety of genetically modified plants ever since the introduction of transgenic plant products into the market. This debate is still very much alive and several issues have been raised, including the safety of transgenic food and the environmental impact of transgenic plants (Schubert 2002; Dale et al. 2002; Liu et al. 2005). In fact, Bt-maize has been found to have unexpected modifications that could exert some effect on soil environment. In Bt-maize, cry protein has been found to be released in the root exudates (Saxena et al. 1999, 2002). Indeed the rhizosphere environment hosts a complex microorganism network which interacts very closely with plant roots, the outcome of such interactions being seen in the strong influence plant type has on the microbial ecosystem of the soil. On considering different exudates pattern of Bt-maize with respect to non-Bt-maize, there was no significant differences in the colony-forming units of culturable bacteria, actinomycetes, fungi, protozoa, nematode in the rhizospheres of transgenic Bt- and non-transgenic Bt-maize (Saxena and Stotzky 2001). Koskella and Stotzky (2002) confirmed that several cry toxins from *B. thuringiensis* have no microbicidal or microbiostatic activities against selected bacteria.
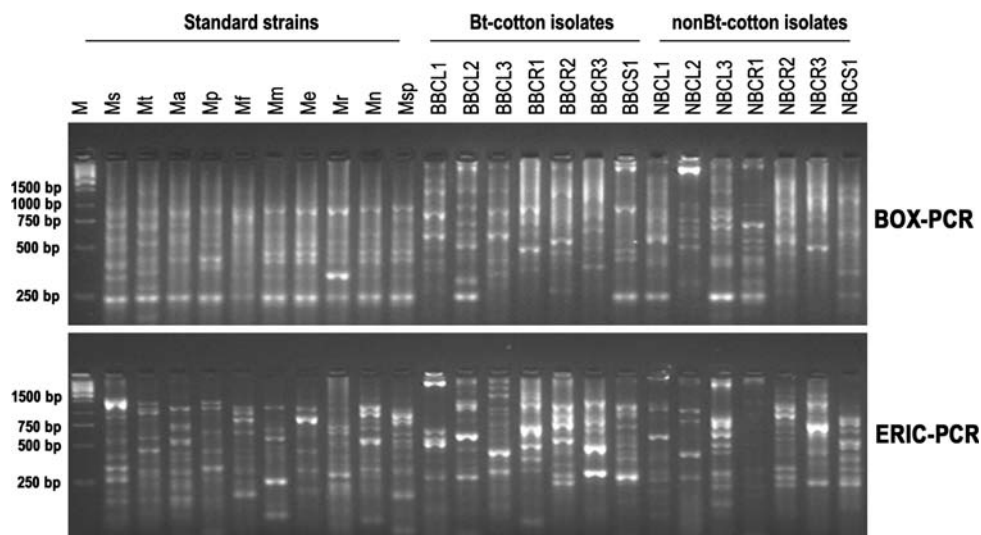
**Fig. 3** BOX (**a**) and ERIC (**b**) fingerprinting of PPFM isolates from Bt-cotton and non-Bt-cotton and standard strains of *Methylobacterium* species. M—1 kb DNA marker ladder; Ms—*M. suomiense* (TNAU4); Mt—*M. thiocyanatum* (TNAU8); Ma—*M. aminovorans* (TNAU 9); Mp—*M. populi* (TNAU12); Mf—*M. fujisawaense* (DSM5686[T]); Mm—*M. mesophilicum* (DSM1708[T]); Me—*M. extorquens* (DSM1337[T]); Mr—*M. radiotolerans* (DSM1819[T]); Mn—*M. nodulans* (ORS2060[T]); Msp—*Methylobacterium* sp. (TNAU14). BBC—Bt-Bunny cotton; NBC—non-Bt-Bunny cotton

In India, the cultivation of Bt-cotton which has increased tremendously every year is considered to be environmentally safe because of the reduced pesticide use. With the rising popularity of Bt-cotton, the safety of Bt-cotton to the biodiversity and other non-targeted organisms is also now in debate. PPFMs are one of the closely associated bacterial communities of cotton, which are found in the phyllosphere, rhizoplane and as endophytes. In the phyllosphere, they colonize primarily the midrib regions and effectively utilize the methanol released from leaves and in turn provide cytokinins to the plant (Madhaiyan et al. 2005). The endophytic PPFMs induce the

systemic resistance of the host plants against phytopathogenic bacteria (Lacava et al. 2004). The diversity of PPFMs in various crops is well-documented by metabolic and molecular techniques by our group already (Balachandar et al. 2008). This investigation was mainly carried out to monitor the changes in the occurrence of PPFMs in leaves, root surfaces and inner tissues of transgenic Bt-cotton. The population dynamics of PPFMs in phyllosphere, rhizoplane and internal tissues of both Bt-cotton and non-Bt-cotton showed no differences. Also the metabolic fingerprinting of PPFM isolates carried out by differential carbon-substrate utilization profile also did not



**Fig. 4** UPGMA dendrogram based on BOX and ERIC fingerprinting of PPFM isolates from Bt-cotton and non-Bt-cotton and standard strains of *Methylobacterium* species. The cluster was built based on the matrix generated with DNA fragments amplified by BOX- and ERIC-PCR. The coefficient used was Jaccard's similarity coefficient. BBC—Bt-Bunny cotton; NBC—non-Bt-Bunny cotton
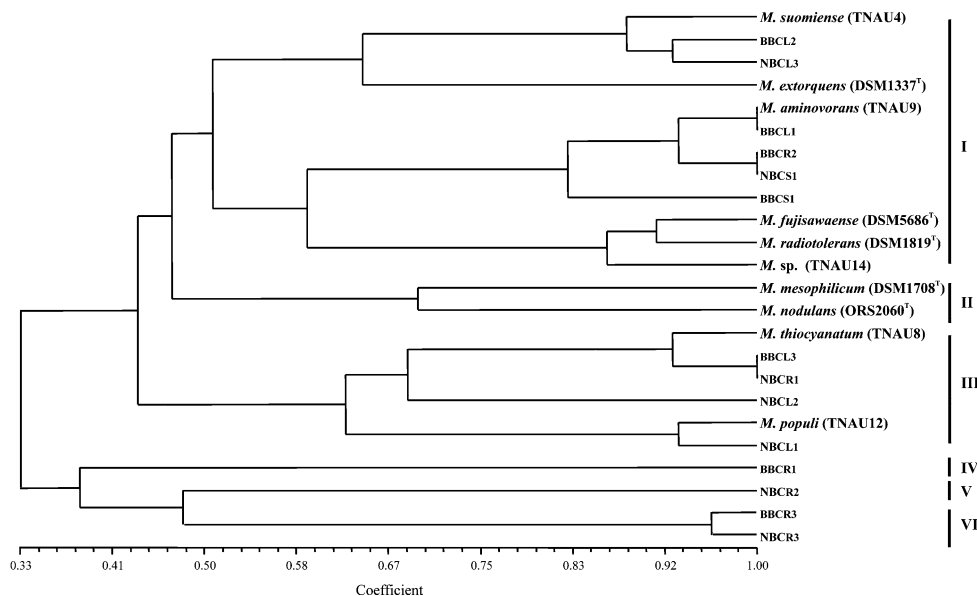
detect differences between Bt-cotton and non-Bt-cotton. Three PPFM groups in phyllosphere and rhizoplane were present in both Bt-cotton and non-Bt-cotton. DNA fingerprinting analysis based on PCR such as ARDRA, RISA, BOX-PCR and ERIC-PCR was performed with Bt- and non-Bt-cotton PPFM isolates. All these results revealed no difference between Bt-cotton and non-Bt-cotton. In both cotton cultivars, the common PPFM isolates such as *Methylobacterium suomiense, M. populi, M. aminovorans* and *M. thiocyanatum* were found to colonize the phyllosphere, rhizoplane and internal tissues of plants.

Rui et al. (2005) reported insignificant functional bacterial population in the rhizosphere between Bt-cotton and non-Bt-cotton. Similarly the metabolic profiling by BIO-LOG carbon-substrate utilization and molecular analysis of 16S rRNA genes by DGGE of rhizospheres of transgenic and non-transgenic corn also revealed no difference in the community structure. However, the difference in diversity of rhizosphere bacterial communities was recorded as being due to different soil textures, not between corn varieties (Fang et al. 2005). Most studies revealed that the Bt-toxins excreted through the root or present in the plant are harmless to most plant-associated microorganisms. In this present study, PPFMs were monitored in phyllosphere, rhizoplane and internal tissues of stem of transgenic cotton plants, where the Bt-toxin accumulation is very high. Even in these locations, there was no alteration in the diversity of the PPFM community, which was confirmed by metabolic fingerprinting and molecular diversity analyses such as ARDRA, RISA, BOX- and ERIC-PCR between isolates. The present results are in agreement with several findings of works reported in different non-target microorganisms and microbial activities such as rhizosphere microflora (Brusetti et al. 2004; Fang et al. 2005), phosphorus- and potassium-solubilizing bacteria, nitrogen-fixing bacteria (Rui et al. 2005) and soil enzymes like urease, phosphatase, dehydrogenase, phenol oxidase and protease (Shen et al. 2006). It is concluded that the plant-associated PPFMs, present in phyllosphere, rhizoplane and internal tissues of cotton, are one of the non-target microbial communities neither affected nor altered by Bt-toxin of transgenic cotton.

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