CONSTRUCTION OF A BINARY VECTOR HARBORING Bt GENE AND ITS TRANSFER TO *Trichoderma harzianum*

Gao Xingxi, Yang Qian and Guo Zhaokui

Department of Life Science and Engineering, Harbin Institute of Technology, Harbin 150001, P R China

ABSTRACT

A binary vector harboring Bt gene was constructed and was transferred into biocontrol fungus *T. harzianum* by *Agrobacterium tumefaciens*-mediated transformation system. Southern blot analysis showed that the Bt gene was integrated into the genome of *T. harzianum,* and the transformants contained single copies of the gene. The transformation efficiency was 50-160 transformants per 10^6 spores.

Keywords: *Agrobacterium tumefaciens*, *Bacillus thuringiensis*, *Trichoderma harzianum*, genetic transformation, plasmid construction

1. INTRODUCTION

Bacillus thuringiensis (Bt) is a species of gram-positive bacteria that produces highly active insecticidal proteins (Knowles, 1994). Bt insecticides, have been used as sprays for more than 50 years (Cannon, 1993), but they have not been widely adopted in large-scale crop protection because of their high cost, narrow spectrum of activity and rapid degradation in the environment. With the advent of genetic engineering, there are new possibilities for the application of Bt insecticides. Obukowicz *et al.* (1986) have transferred Bt gene to a root-colonizing bacterium *Pseudomonous fluorescens*, which not only lengthened the activity of Bt, but also provided the plant with natural protection against soil-dwelling insects. In addition to the transfer of Bt gene to epiphytes, the practice of transferring it to endophytes has also been achieved (Liu *et al.*, 1995). We have constructed a binary vector harboring the Bt gene, and transfered it to another biocontrol agents-filamentous fungi.

Several methods are available for the genetic transformation of filamentous fungi (Yan *et al.*, 1999). Most protocols involve the transformation of protoplasts mediated by polyethylene glycol (PEG) or the use of electroporation. The isolation of protoplasts is not only time-consuming, with low viability, but also tends to result in low transformation efficiency. Until recently, PEG-mediated transformation of protoplast has been the method of choice for filamentous fungi. However, an established method for plant transformation by *Agrobacterium fumefaciens* has been developed for use in Yeast (Bundock *et al.*, 1995), and a number of filamentous fungi (de Groot *et al.*, 1998, Gouka *et al.*, 1999, Abuodeh *et al.*, 2000). Apparently, *Agrobacterium tumefaciens*-mediated transformation method has none of the disadvantages associated with the above-mentioned protocols. This report describes the transformation of *T. harzianum* by *Agrobacterium tumefaciens*-mediated transformation system.

2. MATERIALS AND METHODS

Strains and plasmids

Agrobacterium tumefaciens AGL1 was kindly provided by Prof. Chu Chengcai (The Chinese Academy of Science), *T. harzianum* was from Hebei Agricultural University. Plasmid pFWZ10 containing Bt gene (Fig1) was supplied by the Chinese Academy of Agriculture, binary vector pPK2 (**Fig.1**) was a generous gift from Prof. S.F. Covert (University of Georgia, USA).

Binary vector construction

Recombinant plasmid pPKBt was constructed by insertion of a 2kb HindIII /XbaI fragment from pFWZ10 containing the Bt gene into the same restriction sites of the binary vector pPK2. The recombinant plasmid was transferred into AGL1 by direct transformation method.

Transformation and selection of *T. Harzianum*

Agrobacterium tumefaciens-mediated transformation of *T. harzianum* was carried out as described (S.F. Covert *et al.*, 2001) with the following modifications. The spore suspension was mixed with an equal volume of *A. tumeficiens* culture, prepared as described by Bundock *et al.* (1995). A 200µl aliquot of this mixture was directly plated onto induction medium with 200µM acetosyringone (AS). After 2 d growth, plated again M-100 medium (containing 300µ g/ml cefotaxime and 200µg/ml hygromycin B) onto the induction medium. Putative transformants were visible 5-7 d later.

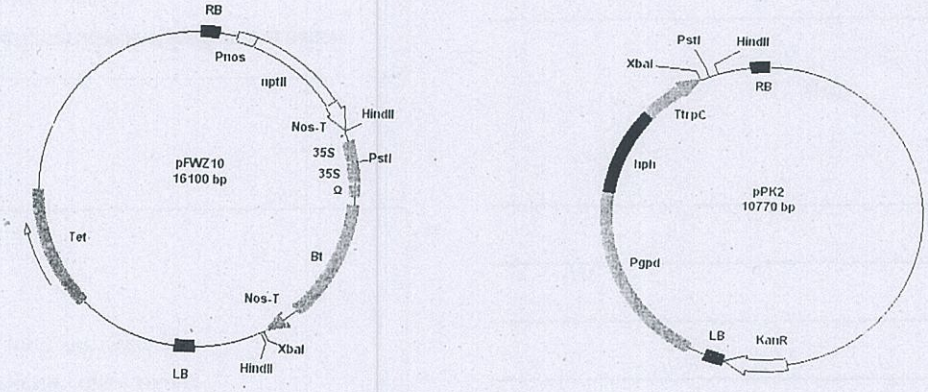

Figure 1. Restriction enzyme map of pFWZ10 and pPK2

Southern blot analysis of fungal transformants

Genomic DNA from *T. harzianum* was isolated as described (Russo *et al.*, 1992).The DNA was digested with EcoRV. The HindIII /XbaI fragment of plasmid pFWZ10 was used to make

probes. DNA gel blot, labelling reactions and hybridization were carried out as described by DIG High Prime DNA Labelling and Detection Starter Kit II (Roche Diagnostics Corporation).

3. RESULTS

Construction and identification of recombinant plasmid

Plasmid pPKBt was constructed by inserting Bt gene from pFWZ10 into pPK2. The recombinant plasmids were extracted after transformation into *E. coli* JM109, and digested with HindIII and HindIII /XbaI respectively. The recombinant plasmid was identified by gel electrophoresis (**Figure 2**).

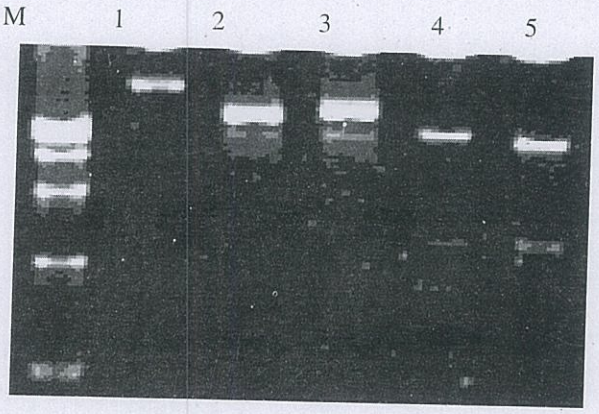

Figure 2. Identification of recombinant plasmid pPKBt by restriction enzymes
M:Marker(DL15000); lane1: pFWZ10; lane2,3:pPKBt;
Lane4:pPK2; lane5:pPKBt digested with HindIII /XbaI

The original sensitivity level of *T. harzianum* to hygromycin

The results (**Table 1**) showed that hygromycin inhibited the growth of *T. harzianum* obviously when its concentration is 100μg/ml. The fungus can not survive when the concentration was up to150μg/ml.

Table 1. Growth inhibition (%) of *T. Harzianum* by hygromycin

Hygromycin concentration ($\mu g.ml^{-1}$)	0	50	75	100	125	150
1	0	40.00	42.86	65.14	88.00	100
2	0	19.34	30.23	49.61	92.25	100
3	0	21.43	37.14	55.71	92.86	100
mean	0	26.92	36.74	56.82	91.03	100
S_{n-1}	0	11.37	6.32	7.82	1.29	0

The resistance level test of *T. harzianum* transformants

The transformants were obtained after 5-7 days cocultivation on M-100 plates containing 300μg/ml cefotaxime and 200μg/ml hygromycin. These transformants grew well when transferred to new plates containing 200μg/ml hygromycin. Further tests on different concentrations of hygromycin showed that the transformants can grow well on all treatments

(**Table 2**). The results of repeated experiments showed the transformation rate was 50-190 transformants per 10^6 spores.

Table 2. Colony growth(cm) of *T. harzianum* transformants at different concentrations of hygromycin

Hygromycin concentration ($\mu g.ml^{-1}$)	0	100	150	200	150	300
1	2.72	2.58	2.60	2.82	2.80	2.54
2	2.60	2.72	2.70	2.80	2.64	2.82
3	2.48	2.60	2.40	2.50	2.50	2.30
mean	2.60	2.63	2.56	2.71	2.65	2.55
S_{n-1}	0.3	0.1	0.2	0.2	0.2	0.3

Southern blot analysis of transformants

DNA gel blots (**Fig. 3**) indicated that Bt gene was actually integrated into the genome of *T. harzianum*. In addition, the results also showed that these transformants contained single copies.

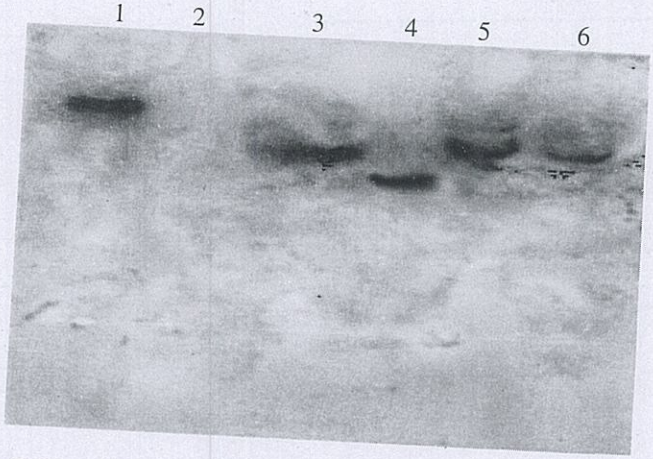

Figure 3. DNA gel blot analysis of the transformants lane 1,pFWZ10; lane 2, wild strain; lane 3-6, transformants

4. DISCUSSION

In this report we described the construction of a novel binary vector harboring Bt gene and its transformation to *T. harzianum* by *Agrobacterium tumefaciens*-mediated transformation method. The Bt gene used in this study was artificially synthesized and modified according to plant codons (Wang *et al.*, 1994), which are highly expressed in plants. The promoter is a doubled CaMV35S, and there is a Ω sequence between promoter and target gene. The enhanced CaMV35S promoter has an excellent performance in plants. CaMV35S promoter can function in some filamentous fungi (Mullins *et al.*, 2000, Hoang *et al.*, 2001). The enhanced promoter used here was expected to function better in *T. harzianum*.

We transferred Bt gene into biocontrol fungus *T. harzianum* in order to construct a genetically engineered strain against not only insect pests but also plant pathogens. Further work is in progress, concerning the expression of Bt gene in *T. harzianum*.

Agrobacterium tumefaciens-mediated transformation system has displayed many advantages, since it is used in the transformation of filamentous fungi. In this study, *T. harzianum* was transformed by this method, and the transformation rate was increased 20-50

folds, compared with previous work (Yang *et al.*, 1998). Southern blot analysis showed that the Bt gene integrated into the genome of *T. harzianum*, and the transformants contained single copies, which was comparable with the report of S. F. Covert (2000) This study also indicated that *Agrobacterium tumefaciens*-mediated transformation system was very effective and convenient for the transformation of T. *harzianum*. Therefore, *Agrobacterium tumefaciens*-mediated transformation system maybe become a powerful tool for the study of filamentous fungi.

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