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Generation of Antibiosis-free Mutants of *Bacillus subtilis* NCD-2 with Transposon Tn917 and the Role of Antibiosis of NCD-2 in Controlling Cotton Sore Shin

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Abstract: The *Bacillus subtilis* NCD-2, isolated from the rhizosphere of cotton, could control cotton *Verticillium* wilt effectively in field. This bacterial strain inhibited the phytopathogen *Verticillium dahliae* and *Rhizoctonia solani*, which caused cotton *Verticillium* wilt and cotton sore shin, respectively, by secreting antipeptides. To determine the role of the antipeptide in controlling cotton disease, the transposon Tn917 insertional mutagenesis was applied to NCD-2 strain. In this study, *B. subtilis* NCD-2 was transformed with a plasmid pTV1 carrying Tn917 by protoplast methods. 1500 mutants were screened against *V. dahliae* and two antibiosis-free mutants were obtained with resistance to erythromycin and lincomycin but susceptible to chloromycetin. The greenhouse experiment on cotton sore shin control with NCD-2 wild stain and antibiosis-free mutants showed that antibiosis of NCD-2 played an important role in controlling this disease. The result suggested that the functional gene of *B. subtilis* NCD-2 encoding the antipeptide against *V. dahliae* had a significant function in controlling of cotton sore shin disease.

Key words: antibiosis; Tn917; mutagenesis; transposon tag; *Bacillus subtilis*; *Verticillium dahliae*

The *Bacillus subtilis* NCD-2, isolated from the rhizosphere of cotton, was an effective bacterial strain to control cotton *Verticillium* wilt in fields[1-2]. It had been proved that NCD-2 strain inhibited the phytopathogen *Verticillium dahliae* which caused cotton *Verticillium* wilt by secreting antipeptides[3]. Genetic analyses have been a key to unravel the biology of microorganisms with transposon mutagenesis. Transposons can generate insertional mutations that were readily mapped, and depending on the particular transposon used, can also create reporter gene fusions at the sites of their insertion[4-5]. The transposon Tn917 was successfully applied to a number of studies. With this method, the mutants of *B. subtilis* and other positive bacterium could be obtained[4, 6-8]. In this study, the transposon Tn917 was inserted into genome DNA of *B. subtilis* NCD-2, and two mutants with antibiosis-free against *V. dahliae* was screened. In addition, the role of gene encoding the antipeptides against *V. dahliae* was determined in controlling the cotton sore shin.

1 Materials and Methods

1.1 Bacterial strains and culture conditions

B. subtilis NCD-2, isolated from the rhizosphere of cotton, provided by the laboratory of corresponding author. *B. subtilis* PY143 was kindly provided by Biocontrol Laboratory of

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Plant Disease, China Agriculture University. *B. subtilis* strains were cultured in Luria-Bertani (LB) medium supplemented when necessary with the appropriate antibiotics (Chloramphenicol 5 g • L^{-1}, Erythromycin 1 g • L^{-1}) and Lincomycin 25 g • L^{-1}). Solid media contained 1.5% agar.

1.2 Enzyme and antibiotics

Restriction endonucleases including PstI, EcoRI and KpnI, were bought from TaKaRa Biotechnology (Dalian) Company (Dalian, China). Erythromycin and Chloramphenicol were bought from Amresco Company. Lincomycin was bought from North China Pharmaceutical Group Corporation.

1.3 Methods for transformation and mutagenesis of *B. subtilis* NCD-2

The transposon Tn917 used in this study was delivered by the pTV1 vector and used for random insertion mutagenesis. The plasmid pTV1 was prepared using PAL method[9] and transformed into *B. subtilis* NCD-2 by protoplast methods[10]. Transformant isolates were screened according to the plasmid-associated properties, i.e., Cm^r and Lin^r at the permissive temperature (30° C) for plasmid replication. The plasmid DNA was extracted from the transformansts and used as the template DNA for PCR verification of the isolates containing the original intact plasmid pTV1. Representative plasmid-containing isolates were induced for transposon using the method described by Youngman et al[5], with the incubating temperature of 50℃, instead of 48℃ in Youngman's method. The mutants were selected based on the phenotype with Erm^r, Lin^r and Cm^s.

1.4 Assay of the transposon Tn917

One pair of primers was designed according to the sequence of erythromycin resistant gene in Tn917: EM189 (5′-CTGGAACATCTGTGGTATGGC-3′) and ERM624 (5′-GGTACAGGGCATTTAACGACG-3′). PCR verification was conducted with these primers to amplify template DNAs extracted from *B. subtilis* NCD-2, transformants and mutants. About 436 bp DNA fragment could be amplified if the template DNA containing the transposon Tn917.

1.5 Screening antibiosis-free mutants against *V. dahliae*

All mutants were tested for their inhibition against *V. dahliae* through dual-layer medium. When the inhibition effect of the mutants changed significantly compared to *B. subtilis* NCD-2, the mutants were screened out.

1.6 The effect of functional gene encoding the antipeptide against *V. dahliae* in *B. subtilis* NCD-2 on controlling cotton sore shin

The *B. subtilis* NCD-2 wild strain, transformants and antibiosis-free mutants were used to determine their controlling efficacy on cotton sore shin. All tested bacterial isolates were incubated in LB culture solution under the condition of 30℃, 160 r • min^{-1} for 24 h, then the culture solution were diluted with sterilized water to contain about 109 bacterial cells per mL. Cotton seeds (cultivar 33B) were soaked for 1 h in the suspension of each bacterial isolate, and the seeds soaked in water were used as control. All treated seeds were sowed in a special fertilizer containing soil (collected from cotton field), vermiculite and chicken feces mixed at the weight proportion 1∶1∶1. The incidence of cotton sore shin was examined when the control reached at the peak incidence rate of this disease. There were three replications for each treatment.

2 Results and Analysis

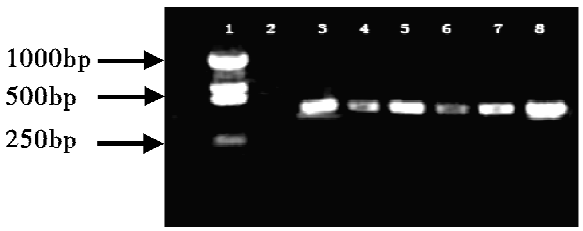
2.1 Tn917 insertional mutant library of *B. subtilis* NCD-2

Twenty transformants resistant to Chloramphenicol were obtained through protoplast-mediated genetic transformation method. The transformants were confirmed through microscope observation and assay of the transposon Tn917 (Lane 1-5 of figure 1). Tn917 was inserted into

chromosome of *B. subtilis* NCD-2 by inducible transposition, and the mutants resistant to erythromycin and lincomycin but sensitive to Chloromycetin were selected. A Tn917 insertional mutant library of *B. subtilis* NCD-2 containing 1500 transposons was obtained.

2.2 Screening antibiosis-free mutants against *V. dahliae*

The 1500 transposants were tested for their inhibition ability against *V. dahliae*, and two antibiosis-free mutants were obtained. They were designated as NCD-2$^-$-16 and NCD-2$^-$-20. A size of about 436 bp DNA Fragment (Lane 6-7 of figure 1) was amplified from the two mutants, same as that from pTV1 carrying Tn917 (Lane 8 of figure 1). However, this DNA fragment could not be amplified from *B. subtilis* NCD-2 wild strain. These results suggested that the change of antibiosis-free mutants' character might be due to the transposon Tn917 insertion into the chromosome of *B. subtilis* NCD-2.



1, Marker;2, NCD-2 wild strain;3, *B. subtilis* PY143; 4-5, transformant;6-7, antibiosis-free mutants;8, pTV1

Fig. 1 PCR identification of Tn917 in different isolates

2.3 The role of functional gene of *B. subtilis* NCD-2 encoding the antipeptide against *V. dahliae* in controlling cotton sore shin

Four bacterial strains including two antibiosis-free mutants (NCD-2$^-$-16 and NCD-2$^-$-20), *B. subtilis* NCD-2 wild strain (NCD-2) and transformant (NCD-2^t) were tested in this experiment. The results (Table 1) showed that: 1) The effects of four treatments on emergence of cotton seed were not significantly different compared to the control at $P=0.05$ level. 2) The four treatments could significantly reduce the dead seedling percentages compared to the control at $P=0.05$ level, and the percentages were not significantly different between NCD-2 wild strain and the transformant (NCD-2^t), and between two antibiosis-free mutants. However, the dead seedling percentages of two antibiosis-free mutants were significantly higher than NCD-2 wild strain and the transformant. The control efficacies of transformant NCD-2^t and NCD-2 wild stain on cotton sore shin were 84.1% and 76.2%, respectively, while the efficacies of two antibiosis-free mutants were only 33.3% and 44.4%, respectively. These results indicated that the antipeptides secreted by *B. subtilis* NCD-2 played an important role in controlling cotton sore shin disease, meanwhile it was also proved that the genes encoding the antipeptides had an important effect in control of this disease by *B. subtilis* NCD-2.

Table 1 Experiment on the role of functional gene encoding antipeptide against *V. dahliae* in *B. subtilis* NCD-2 in controlling cotton sore shin with *B. subtilis* NCD-2

Treatment	Inhibition ability against *R. solani*	Emergence percentage /%	Dead seedling percentage /%	Control efficacy /%
Control (water)	Negative	61.57ab	24.71a	-
NCD-2 (wild strain)	Positive	62.74ab	5.88c	76.2
NCD-2^t(transformant)	Positive	60.00ab	3.92c	84.1
NCD-2-16 (antibiosis-free mutants)	Negative	63.50a	16.47b	33.3
NCD-2$^-$-20 (antibiosis-free mutants)	Negative	56.08b	13.73b	44.4

Note: data in the same column followed with same letters were not significantly different at level of $P=0.05$.

3 Discussion

The copy number of the plasmid pTV1 in *Bacillus subtilis* PY143 was very low, so it was difficult to extract and purify the plasmid pTV1 from PY143. In this study, different methods

for extraction and purification with this plasmid were compared and the PAL method was recognized as the optimized one.

It was extremely difficult and even impossible in some case to transform some *Bacillus subtilis* strains with the plasmid pTV1 carrying transposon Tn917[11], therefore, the transformation of *Bacillus subtilis* NCD-2 was key procedure for this study. Four methods including protoplast methods, electro-transformation, chlorination calcium, and competent method had been used in this study for transformation of *B. subtilis*NCD-2 with plasmid pTV1, but only withprotoplast method some transformants were successfully obtained. The reasons for this result would be studied in future experiments.

It was found that the plasmid extracted from transformants could not be digested by corresponding restriction endonuclease, so it was uncertain that the plasmid extracted from the transformants was pTV1. However, the positive evidence was found by PCR to amplify the DNA fragment of Erythromycin gene in Tn917 carried by pTV1. Why the plasmid could not be digested by corresponding restriction endonuclease needs further study.

To induce the transposition of transposon Tn917 with Youngman method, the key was to treat transformants with consecutive high temperature (48～52℃), or successful transposition could not be achieved. This study showed that the transposition efficiency of Tn917 was higher at 50℃ than at 48℃.

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