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Full Length Research Paper

Using a sequence characterized amplified region (SCAR) marker for detection of *Bacillus* strain TS02 sprayed on strawberry plants to bio-control powdery mildew in fields

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This work used sequence characterized amplified region (SCAR) marker to detect the *Bacillus cereus* strain in strawberry fields. The purpose was to develop an effective molecular method for detecting the functional target microorganisms applied in agricultural fields. A 3×10^9 CFU/ml vegetative cell suspension based on the functional *B. cereus* strain TS02 was sprayed on strawberry plants to control powdery mildew. Primer pair LS400: 5'-TCC AAC TAC TTC TCC AT-3' and 3'-TTT GCC ATT ACA TAG AGT-5' was used to amplify the 400 bp SCAR marker TSS_1. TSS_1 could detect TS02 specifically, in relation to other 7 *Bacillus* species and 6 *B. cereus* strains. Sensitivity detection of TSS_1 to the lowest DNA concentration of TS02 was 78 pg/µl, which corresponded to a density of 8×10^5 CFU/ml cells of strain TS02. TS02 was specifically detected by TSS_1 from the collected mixture of microorganisms on leaves during the days 1 - 12 after TS02 was sprayed in strawberry fields. TS02 DNA concentration reached about 2500 pg/µl during the days 5 - 7 after spraying and 156 pg/µl in the final day 12, which corresponded to a density of about 2.5×10^7 and 1.6×10^6 CFU/ml cells of TS02 respectively. These results showed that SCAR marker TSS_1 is an effective method for the detection of strain TS02 after applied in fields. This research provided a simple and convenient method of detection of target microorganisms in fields, It could especially help monitor strain TS02 more effectively when widely been applied in agriculture.

Key words: *Bacillus cereus* strain, biocontrol, sequence characterized amplified region marker, strawberry, *Sphaerotheca macularis.*

INTRODUCTION

Powdery mildew of strawberry, caused by *Sphaerotheca macularis*, has recently become a severe problem in most areas of the world where strawberries are grown. Major epidemics reduce crop yields by causing decreased fruit set, inadequate ripening, fruit cracking and deformation. It can also cause poor flavor development and reduce post-harvest storage time. Development and spread of powdery mildew is favored by moderate to high humidity and temperature, so it is typically more severe in protected planting. Strawberry cultivars differ widely in their resistance to powdery mildew, but the most popular cultivars are quite susceptible to the disease (Maas, 1998; Zhang et al., 2004).

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Abbreviations: SCAR, sequence characterized amplified region; **BCAs,** bio-control agents; **PCR,** polymerase chain reaction; **NB,** nutrient broth.

The production of strawberries is largely dependent on using a number of chemicals to control *S macularis*. The fungicide treatments against powdery mildew are required much more under protected planting for each growing cycle. Pesticide residues are becoming a major issue, resulting in legislative actions to limit and regulate pesticide. As a result, new alternatives, such as bio-control agents (BCAs), are required to reduce pesticide residues on fruits. Some isolates of *Bacillus* sp. are reported as growth promoters or antagonists of several pathogens in the microbial products. The antagonists studied are mainly *Bacillus subtilis* and *Bacillus thuringiensis*, occasionally *Bacillus licheniformis, Bacillus brevis, Bacillus polymyxa, Bacillus cereus and Bacillus pumilus* (Felici et al., 2008b; Brooks et al., 1994; Obagwu and Korsten, 2003; Bobrowski et al., 2002; Dhillon and Sharma, 2009; Wang et al., 2008; Haggag, 2008; Selvakumar et al., 2007; Mari et al., 1996). The number of available BCAs of strawberry powdery mildew is limited (Pertot et al., 2008). There are very few research papers that have reported *B. cereus* as BCAs to bio-control strawberry powdery mildew in fields (Chen, 2008; Chen et al., 2007).

A series of TS strains have been separated in our laboratory from 2006, all of them are *Bacillus* strains, some of them have been tested to be the antagonist strains against plant pathogens. For example, the *B. licheniformis* strain TS01 is an antagonist strain against *Alternaria mali* (Wang et al., 2008) in apple, the *B. cereus* strain TS02 is an antagonist strain against *S. macularis* in strawberry (Chen, 2008; Chen et al., 2007), and the *B. subtilis* strain TS06 is an antagonist strain against *Fusarium oxysporun* in strawberry (Li, 2009a; Li et al., 2009b). A novel bio-control agent, based on strain TS02, is used as an alternative strategy for strawberry powdery mildew management in China Agricultural University (CAU) agricultural research center throughout the growth period. According to the standard field experimental rule of pesticide controlling effect of the People's Republic of China, the effects of TS02 strain on strawberry powdery mildew had been tested in fields for several years. The results indicated that TS02 strain could control strawberry powdery mildew. A live bacteria concentration above 3×10^7 CFU/ml could achieve an ideal effect which was the combined result of live bacteria and the secretions of bacteria.

For the valid application of bio-control agents in agriculture, the unambiguous detection of bio-control micro-organisms is a prerequisite. Traditional methods based on phenotypic patterns for detection of target micro-organisms in BCAs lack specificity and can introduce bias due to over or under estimation of the microorganisms studied; it is also time consuming (Matsuki et al., 2003; Badosa et al., 2004). Specific compounds can be detected in targeted microorganisms but this approach tends to be too complex for routine and high throughput analysis (Van der Zee et al., 1997; Hood, 1999). For the last 10 years, polymerase chain reaction (PCR)-base amplification strategies have been extensively studied for the detection of microorganisms (Ward and Roy, 2005). Converting random amplified polymorphic DNA (RAPD) markers into SCAR markers that are specific for a given genome has been demonstrated to be a useful molecular tool. SCAR markers can be diagnostic for a genus, species, or even a particular strain (De Clercq et al., 2003; Pujol et al., 2005; Felici et al., 2008a). The development of SCAR markers to detect bio-control micro-organisms has been reported for bacteria (Broggini et al., 2005), fungi (Schena et al., 2002; Dauch et al., 2003) and yeasts (De Clercq et al., 2003). However, so far this approach is mostly researched under controlled conditions, and has not been developed for detecting *B. cereus* strains in strawberry fields (Chen, 2007).

In this work, our research focused on the *B. cereus* strain TS02. The aim is to develop an effective and convenient molecular method for the detection of the target microorganism strains, especially when they are applied widely in agricultural fields.

MATERIALS AND METHODS

Bacterial strain

B. cereus strain TS02, isolated and selected from soil, was identified as having the effects to bio-control strawberry powdery mildew. Before application in fields, strain TS02 was grown in nutrient broth (NB) liquid medium at 37°C and vibrated on 220 g/min for 24 h to get the vegetative cell suspensions. Vegetative cell suspensions were then diluted in order to obtain the desired bacterial concentration (3×10^9 CFU/ml) for treatment of strawberry plants.

Specificity identification of SCAR marker TSS_1 to strain TS02

A specific sequence of 513 bp RAPD marker TSR_1 was amplified by the G09 random primer, and it had been selected and identified as the unique RAPD fragment of strain TS02 when compared to other tested strains of the species of *Bacillus* (Chen, 2008). Based on the 513 bp sequence of RAPD marker TSR_1, a developed primer pair LS400 (5'-TCC AAC TAC TTC TCC AT-3' and 3'-TTT GCC ATT ACA TAG AGT-5') was designed to amplified a 400 bp band and was then selected to be converted into SCAR markers TSS_1 for the specificity identification.

DNA of strain TS02 and other strains tested in Table 1 were extracted after being consecutively cultured in NB liquid medium overnight at 37°C. Qiagen DNA Mini Kit was used and extraction steps were according to the manufacturer's instructions. PCR amplification tests were performed to assess the specificity of SCAR marker TSS_1 to strain TS02 by using the primer pair LS400 amplified on the DNA from cultures of strain TS02 and other strains. The PCR mixtures consisted of a final volume of 50 µl of reagents and PCR amplification was carried out in a Geneamp 9600 programmable thermocycler. The 50 µl PCR mixtures contained the following reagents: 200 µmol/l dNTP, 2 mmol/l Mg^{2+}, 20 pmol/l of each primer, 1.5 u Pfu polymerase and 10 × PCR buffer 10 µl. The reaction conditions were a denaturing step of 94°C for 2.5 min followed by 35 cycles of 94°C for 45 s, 52°C for 1 min and 72°C for 1.5 min, finished with 72°C for 10 min and held at 4°C. The PCR

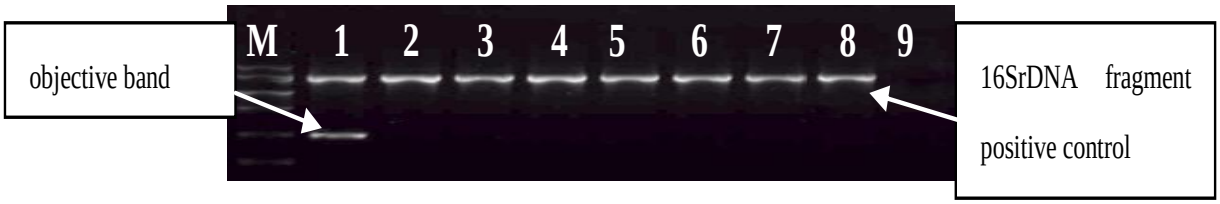

Figure 1. PCR amplified results of different specific bacteria DNA with SCAR marker TSS_1. M: marker ; Lanes 1 - 8: *Bacillus cereus* TS02, *Bacillus licheniformis* 14580, *Bacillus licheniformis* B-402-1, *Bacillus subitilis* subsp. *Subtilis* 6051-U, *Bacillus subitilis* 6633, *Bacillus coagulans* 7050, *Bacillus megaterium* 14581, *Bacillus pumilus*7061; Lane 9: negative control.

Figure 2. PCR amplified results of different *Bacillus cereus* bacteria DNA with SCAR marker TSS_1. M: Marker; Lanes 1 - 7: *Bacillus cereus* TS02, *Bacillus cereus* 10117, *Bacillus cereus* 10602, *Bacillus cereus* 10603, *Bacillus cereus* 10605, *Bacillus cereus* 10606, *Bacillus cereus* 10607; Lane 8: negative control.

products were visualized by staining with ethidium bromide after electrophoresis on 1% agarose gels in 0.5 x TBE. For veracity, a multiplex PCR was employed with two primer pairs to amplify the SCAR marker TSS_1 and 16S rDNA fragment synchronously in one PCR reaction. The 16S rDNA fragment was the positive control and amplified with the general prime pair 16SF: 5'- AGT TTG ATC CTG GCT CAG -3'and 16SR: 5'- GGT TAC CTT GTT ACG ACT T -3'. The non-DNA sample was the negative control.

DNA collection and preparation of mixtures on strawberry leaves after strain TS02 was applied in fields

Field trials were conducted at Agricultural Research Center of CAU in Beijing. Strawberry cultivar Toyonoka (15 cm between plants, 60 cm between rows) was used. Strain TS02 was applied as 3×10^9 CFU/ml vegetative cell suspensions to strawberry plants using a conventional orchard sprayer. Two sprays were made at growth periods (25 May and 6 June), the interval time was 12 days. Leaf samples were taken at 1-day time intervals and 12 samples were taken in total. Leaves were collected and cut in 0.1 cm pieces with sterile shears and put in 1.5-ml eppendorf tubes. The samples were washed by adding 100 µl sterile distilled water and shaken vigorously for 30 s. 20 µl of the resulting wash solution was transferred to 200 µl of NB liquid medium and consecutively grown overnight at 37°C. Qiagen DNA Mini Kit was used and DNA extraction steps were according to manufacturer's instructions.

Using SCAR marker TSS_1 to detect the dynamic changes of strain TS02 applied in fields

SCAR marker TSS_1 was used to detect the dynamic changes of strain TS02 by amplification of the DNA collections from the strawberry leaves after TS02 applied in fields. The primer pair LS400 was used to amplify TSS_1 marker. The program and system of polymerase chain reactions were same as the aforementioned in "specificity identification of SCAR marker TSS_1 to strain TS02".

RESULTS

Specificity identification of SCAR marker TSS_1 to strain TS02

The specificity of SCAR marker TSS_1 to strain TS02 was assessed against pure cultures of the type bacterial strains (Table 1). DNA samples from 6 strains of *B. cereus* and 7 species belonging to the *Bacillus* genus were tested with primer pair LS400. The expected 400 bp SCAR marker TSS_1 was observed and amplified only in strain TS02, other bacterial strains did not show any PCR product (Figures 1 and 2). The results showed that SCAR marker TSS_1 can detect strain TS02 specifically at species (Figure 1) and strain (Figure 2) level.

The specificity of SCAR marker TSS_1 to strain TS02 was assessed against the mixture samples of the tested strains in Table 1. The results showed that there was no 400 bp TSS_1 marker amplified in the non-TS02 mixture samples, and TSS_1 was detected in the mixture samples with TS02 strain. These results indicated that the TSS_1 marker could detect strain TS02 specifically in the mixture samples of *Bacillus* strains, even though there was an interaction of different *Bacillus* strains in the mixture samples. The specificity of SCAR marker TSS_1 to strain TS02 was also assessed against the field samples collected before or after TS02 spraying on strawberry leaves; the results were same as in the mixture samples of *Bacillus* strains, and are shown in Figure 3. There was no 400 bp TSS_1 marker amplified in the samples before TS02 spraying in fields, and TSS_1 was detected after TS02 spraying. These results indicated that the TSS_1 marker could detect strain TS02 specifically in the field

Table 1. Strains tested and their resources.

Species or strain	Accession no.	Resource
Bacillus cereus	TS02	Our Laboratory
Bacillus cereus	10117	ACCC[a]
Bacillus cereus	10602	ACCC
Bacillus cereus	10603	ACCC
Bacillus cereus	10605	ACCC
Bacillus cereus	10606	ACCC
Bacillus cereus	10607	ACCC
Bacillus coagulans	7050	ATCC[b]
Bacillus megaterium	14581	ATCC
Bacillus licheniformis	14580	ATCC
Bacillus licheniformis	B-402-1	CGMCC[c]
Bacillus subitilis subsp. *subtilis*	6051-U	ATCC
Bacillus subitilis	6633	ATCC
Bacillus pumilus	7061	ATCC

[a]ACCC, Agricultural Culture Collection of China; [b]ATCC, American Type Culture Collection; [c]CGMCC, China General Microbiological Culture Collection Center.

Figure 3. PCR amplified results of total microorganism DNA on strawberry leaf with SCAR marker TSS_1. M: Marker; Lane 1: PCR amplified with the total microorganism DNA on leaf after TS02 spraying in fields; Lane 2: PCR amplified with the total microorganisms DNA on leaf before TS02 spraying in fields; Lane 3: negative control.

conditions.

Sensitivity detection of DNA concentration of SCAR marker TSS_1 to strain TS02 in lab

A series of different 2x diluted DNA concentration of strain TS02 were used to detect the sensitivity of SCAR marker TSS_1. The results showed that the lowest DNA concentration detected was about 78.13 pg/µl (Figure 4). According to a calibration curve between the DNA extracted concentration and cultured cell density that was established in our laboratory (Chen et al., 2007), the lowest DNA concentration 78.13 pg/µl corresponded to a density of about 8×10^5 CFU/ml cells of strain TS02. The calib-ration curve was based on a correlation of DNA extracted concentration and cultured cell density. The detection level was determined by inoculating 200 µl of NB enrichment broth with a dilution series of strain TS02 spores and consecutively grown overnight at 37°C.

Detection of TS02 dynamic changes on strawberry leaves in fields using SCAR marker TSS_1

The average concentration of strain TS02 vegetative cell suspensions sprayed on strawberry plants amounted to 3×10^9 CFU/ml. DNA mixtures of the unknown micro-organisms on leaves were collected and prepared during days 1 - 12 after treatment. The existence and dynamic changes of strain TS02 were detected by using SCAR marker TSS_1. PCR inhibition was observed when attempting to detect directly from leaf washings. Consequently, a culture enrichment step in NB broth prior to analysis was established for strain TS02 detection. The results (Figure 5) showed that strain TS02 was specifically amplified by the SCAR marker TSS_1, which suggested the existence of strain TS02 during days 1 - 12 after spraying. The dynamic changes of strain TS02 had been observed in two stages in fields: TSS_1 bands were getting stronger during the days 1 - 6, and getting weaker during days 7 - 12. DNA concentrations of strain TS02 during 5 -

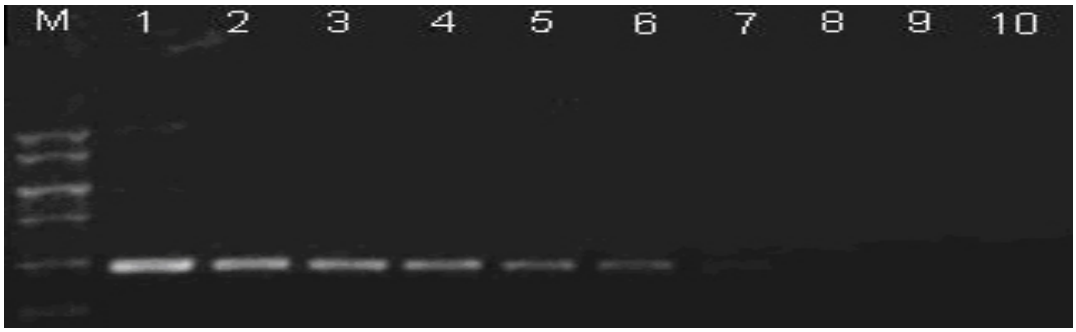

Figure 4. Sensitivity detection of DNA concentration of SCAR marker TSS_1 to the strain TS02. M: Marker; Lanes 1 - 9 DNA concentration (pg/µl) were: 2500, 1250, 625, 312.5, 156.25, 78.13, 39.06, 19.53 and 9.77; Lane 10: negative control.

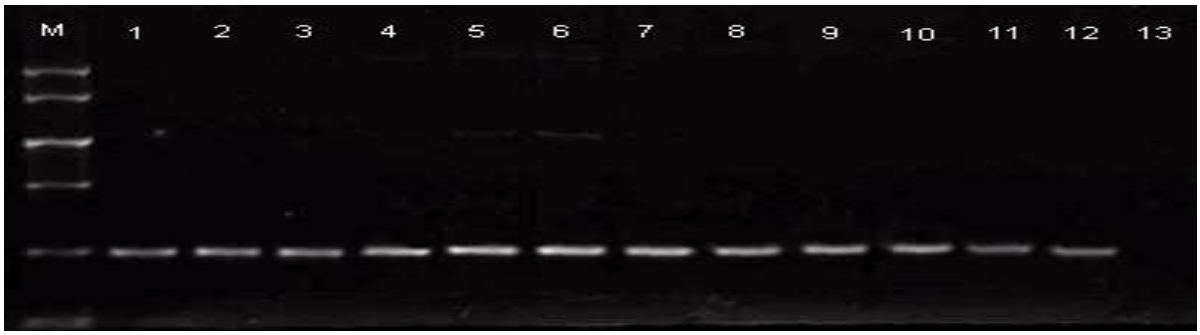

Figure 5. Detecting strain TS02 dynamic changes on strawberry leaf with SCAR marker TSS_1. M: Marker; Lanes 1 - 12: days of TS02 sprayed on leaf in fields; Lane 13: negative control.

7 days reached a high level which was about 2500 pg/µl, which corresponded to a density of about 2.5×10^7 CFU/ml cells of strain TS02. In the final day 12, DNA concentration of strain TS02 was about 156 pg/µl, which corresponded to about 1.6×10^6 CFU/ml of cells of strain TS02. The dynamic changes of strain TS02 after spraying on strawberry plants showed that strain TS02 could live epiphytically in fields for a long time. However, based on preliminary experiments, this depends on environmental conditions. Over 40°C high temperature and very dry field conditions will distinctly decrease the quantity of strain TS02 in fields.

DISCUSSION

A reliable and convenient method for detecting target microorganisms is very important to ensure that BCAs products are applied availably in agricultural fields. Since the number of target microorganisms may change and become very low after BCAs are applied in fields, the detecting method should be sensitive enough to detect target microorganisms as quickly as possible. In the last decade, there has been increasing research attention towards the optimization of PCR-based molecular techniques aimed at the selection of unique and discriminative genomic regions (Lynch et al., 2004), and which made the detection of target microorganisms more sensitive. In general, the characteristic profiles obtained by RAPD technique can potentially be utilized for detection, but RAPD is prone to poor reproducibility. This technique must be performed under very strictly controlled conditions. For the detection of target microorganisms, RAPD markers are normally converted into SCAR markers, because SCAR markers are more specific as they target a known sequence (Pujol et al., 2005). A developed primer pair LS400 was used to amplify the SCAR marker TSS_1 in this work. The strategy of SCAR marker TSS_1 had overcome the poor reproducibility of the RAPD technique successfully in this trial. It has shown that sensitively to the level of 78.13 pg/µl DNA concentration could be detected. The level of 78.13pg/µl DNA concentration corresponded to a density of about 8×10^5 CFU/ml cells of strain TS02.

The major limitation was the inherent biasness of the technique in this work. Every step of the process, including biological, chemical or physical in the analysis of environmental samples, might be a source of bias, potentially distorting the pictures obtained. The greatest hazard in researching the target microorganisms of mixed populations, obtained from the leaf surface, was the insufficient DNA sample. In this work, PCR inhibition was

observed when attempting to detect strain TS02 directly from the leaf washings. Consequently, a culture enrichment step in NB broth prior to DNA extraction was established for the detection. The detection limit was determined by inoculating 200 µl of NB enrichment broth with a dilution series of strain TS02 spores and consecutively grown overnight at 37°C. The culture enrichment step would increase the time spent on the detecting work, but it could ensure that the detected DNA was coming from the live microorganisms in fields. Otherwise, a number of strategies are available to reduce the trial biasing of PCR procedures, e.g. incorporating PCR facilitators in the reaction or controlling the PCR conditions, such as number of cycles and elongation time. The results suggested that all the optimization steps were necessary to make the amplified profiles unchanged in the reaction conditions.

SCAR marker TSS_1 based on the discriminative sequence region of strain TS02 was the guarantee to detect the existence and dynamic changes of strain TS02 in fields. *Bacillus* strains are common in agricultural fields, the fact that no cross-reactions in the mixture samples were detected insured the strain-specific detection of strain TS02 in mixture samples by the SCAR marker TSS_1. This bolsters the use of this method specifically for monitoring the population dynamics of the introduced strains in fields. Further works are needed to test more *Bacillus* strains and study the concentration on different parts of plant at different times in fields.

The detection of dynamic changes of strain TS02 might be used to determine the time of spraying agents in fields. For example, according to our study on the effects of strain TS02, 3×10^7 CFU/ml or over 3×10^7 CFU/ml, TS02 vegetative cell suspension was the effective quantity level of strain TS02 to control strawberry powdery mildew in fields. The results showed that the quantity of strain TS02 on leaf decreased to about 1.6×10^6 CFU/ml cells in the final day 12, and indicated that a new application of spray was needed.

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