ONE-STEP DETECTION OF *CLAVIBACTER MICHIGANENSIS* SUBSP. *MICHIGANENSIS* IN SYMPTOMLESS TOMATO SEEDS USING A TAQMAN PROBE

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SUMMARY

Clavibacter michiganensis subsp. *michiganensis* (CMM), the causal agent of bacterial canker of tomato, is a quarantine organism in many countries. In this study, a one-step method was developed for the detection of CMMs in symptomless tomato seeds directly using a TaqMan probe. The absolute sensitivity threshold was approximately 10 CFU/ml. In seeds the bacteria can be detected in 2 h without DNA extraction when the positive rate is higher than 1%. The method proved specific for CMM and did not react with 11 other plant pathogenic bacterial species or subspecies. Further validation steps using this method for routine detection of CMM are in progress.

Key words: Tomato, *Clavibacter michiganensis* subsp. *michiganensis*, detection, TaqMan probe.

INTRODUCTION

Clavibacter michiganensis subsp. *michiganensis* (CMM) the causal agent of bacterial canker of tomato, is a quarantine organism in Europe (Smith *et al.*, 1997). It is one of the most difficult tomato diseases to control and spreads by infected commercial seed lots and seedlings. The use of healthy seed is the most effective means of control (Chang *et al.*, 1991; Burokiene, 2006). Conventional methods used for detection and identification of the pathogen include biochemical tests, serological assays, and fatty acid and metabolic profiling. These methods, however, have several limitations including poor sensitivity, lack of specificity and long waits for results (Deleon *et al.*, 2006). The first PCR-based assays for detection of infected plants and seeds were described over ten years ago (Dreier *et al.*, 1995). Nevetheless, few diagnostic laboratories have adopted the PCR gel electrophoresis method for routine pathogen identification and disease diagnosis because it is time-consuming, labor intensive and sometimes produces false-positive results. The TaqMan probe PCR method is based on the hybridization of a fluorescent oligonucleotide probe with a specific region within the target that is amplified by traditional primers. Several real-time PCR assays have been developed to detect plant bacteria including *Clavibacter michiganensis* subsp. *sepedonicus* and *Ralstonia solanacearum* in potato tubers (Schaad *et al.*, 1999; Weller *et al.*, 2000). *Xanthomonas oryzae* pv. *oryzae* in naturally infected rice seeds (Xiaolan *et al.*, 2003) and *Xylella fastidiosa* in grapevines (Schaad *et al.*, 2002). The goal of this study was to develop a one-step PCR method using TaqMan probe for the rapid, sensitive and accurate detection of CMM in apparently healthy tomato seed.

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MATERIALS AND METHODS

Bacterial strains and DNA extraction. The bacterial strains used are listed in Table 1. Strains were cultured for 3 days on nutrient dextrose agar (NDA). Genomic DNA was extracted as previously described (Frederick *et al.*, 1998) and DNA concentration measured with a Beckman Du series spectrophotometer.

PCR primers and TaqMan probe. Primers and probe were designed with Primer Express 2.0 software (Applied Biosystems, Foster City, CA, USA) based on the conserved sequence of the ITS gene with an expected length of 270 bp. The primers were ITSYG-1: 5'-CGCGTCAGGCGTCTGTT-3' and ITSYG-2: 5'-AGTGGACGCGAGCATC-3'. The probe was 5'-TGGCGGTGGCGCTCATGG-3', with the 5' end labeled with 6-Carboxyfluorescein (FAM) and the 3' end labeled with tetramethycarboxyrhodamine (TAMRA). The primers and probe were all synthesized by Takara Bio Inc. (Takara, Shiga, Japan).

PCR assays were done on a PE 7700 DNA sequence detection system (Applied Biosystems, Foster City, CA, USA) and MJ PTC 200 DNA Engine Cycler. All reactions were carried out in a final volume of 25 µl containing: 2.5 µl 10×PCR buffer, 3 mM $MgCl_2$, 0.5 mM dATP, dCTP, dTTP, dGTP, 0.4 µM forward and reverse primers, 0.8 µM probe, 1 U *Taq* polymerase and 1 µl DNA template. Sterile molecular biology grade water

Table 1. Bacterial strains used.

No.	Bacterial species[a]	Isolate code
1	*Agrobacterium tumefaciens*	ATCC 27912
2	*Clavibacter michiganensis* subsp. *michiganensis*	APQ0015A
3	*C.m.* subsp. *michiganensis*	APQ 0015B
4	*C.m.* subsp. *michiganensis*	APQ 0015C
5	*C.m.* subsp. *michiganensis*	APQ 0015D
6	*C.m.* subsp. *michiganensis*	APQ 0015E
7	*C.m.* subsp. *michiganensis*	APQ 0015F
8	*C.m.* subsp. *michiganensis*	APQ 0015G
9	*C.m.*. subsp. *insidiosus*	ATCC 10253
10	*C.m.* subsp. *nebrasken*sis	ATCC 27822
11	*C.m.* subsp. *sepedonicum*	ATCC 2140
12	*Erwinia amylovora*	ATCC 15580
16	*Erwinia carotovora* subsp. *carotovora*	ATCC 15713
14	*Pantoea agglomerans* pv. *herbicola*	ATCC 21433
15	*P. ananas* pv. *uredovora*	APQ 0392
13	*P. stewartii* subsp. *stewartii*	ATCC 29229
12	*Rathayibacter tritici*	ATCC 11403
17	*Pseudomonas syringae* pv. *phaseolicola*	ATCC 11365
19	*Xanthomonas oryzae* pv. *oryzae*	ATCC 35933
20	*Xanthomonas oryzae* pv.*oryzicola*	APQ 0333

[a] All strains were obtained from the Institute of Animal and Plant Quarantine

was added to a total volume of 25 µl. Each PCR reaction included a blank control. Reactions were run for 40 cycles as follows: 95°C for 10 min, 94°C for 15 sec, and 60°C for 1 min.

RESULTS

Specificity of the TaqMan probe: To determine the specificity of the TaqMan probe 20 bacterial samples were used. They represented nine strains of *Clavibacter*, three strains of *Pantoea,* two strains each of *Erwinia* and *Xanthomonas*, single strains of *Agrobacterium, Pseudomonas* and *Rathayibacter.* With the TaqMan probe, strong fluorescence signals (Rn value about 9000) could be collected only from the CMM strains. Signals from the other bacteria including the different subspecies of *Clavibacter michiganensis* were on the baseline. These results indicated that the probe can distinguish CMM from other subspecies of *Clavibacter michiganensis*. Seven strains of CMM from different regions were used to further validate the specificity. All were successfully detected.

Sensitivity of the TaqMan probe. A pure culture suspension of 10^8 CFU/ml was diluted eight times in a 10-fold series. Ten µl of the dilutions which contained 1000, 100, 10 and 1 CFU were used as template directly for the test. The template containing 10 CFU gave a signal with Rn value 600. However, one CFU showed no signal. Thus, the detection threshold of this method with a pure culture in buffer suspension is approximately 10 CFU. As the bacteria are transmitted through tomato seeds, we tested to see whether soaking seed in the suspension would decrease detection sensitivity. Fifty healthy tomato seeds were soaked in 2 ml of each dilution for 12 h at 4°C, and 10 µl of these suspensions were then tested by real-time PCR. The signal from the suspension containing 10 CFU was approximately 450, lower than that of the original equivalent dilution (about 600). This was most likely due to a PCR inhibitor in the seeds.

Detection of CMM from tomato seeds. Ten g of naturally infected tomato seeds (detected by ELISA) were soaked in 0.01% Tween-20 overnight at 4°C. The soaking solution was centrifuged for 10 min at 12,000*g* and the pellet resuspended in 10 µl of sterile water, 1 µl of which was assayed by PCR. Strong signals were obtained. Seeds contaminated by different levels of CMM were prepared by mixing 10 infected seeds to 100 and 1000 healthy seeds, and samples were assayed by one-step real time PCR. All samples gave positive results. The signal from seeds with 1% contamination was about 1500 (Fig. 1), indicating that this pathogen can be detected by one-step RT-PCR method in 2 h.

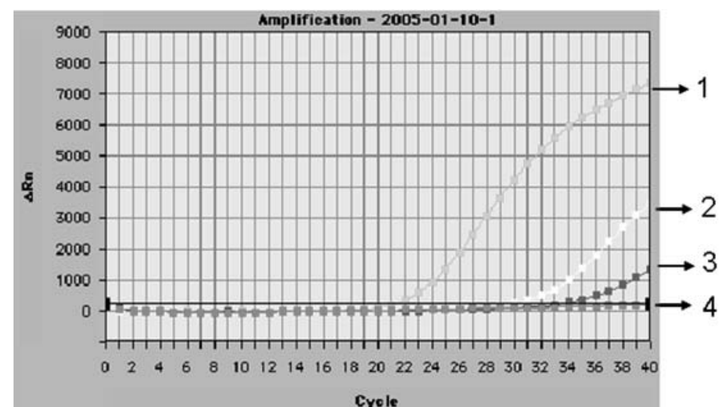

Fig. 1. Seeds with different contamination levels directly detected with the TaqMan probe. 1, naturally infected seeds; 2, 10 infected seeds in 100 healthy ones; 3, 10 infected seeds in 1000 healthy ones; D, healthy seeds.

DISCUSSION

CMM poses a direct threat to production of tomato, one of the most important vegetables in China. Control of this agent ultimately depends on accurate, specific and sensitive detection. Conventional PCR methods have been used to detect this pathogen but real-time PCR offers several advantages such as: less risk of PCR contamination, faster, and quantitative results and potentially higher sensitivity (Dreier *et al* ., 1995; Schaad *et al*., 1999; 2002). In this assay, we were able to test seeds directly without extracting DNA and adding reagents against en-

dogenous PCR inhibitors. This saves time and money, and reduces the chances of cross-contamination of samples. The method was highly specific and did not react with other bacteria tested. The absolute sensitivity threshold was found to be approximately 10 CFU when using a pure culture in buffer solution. In tomato seeds the bacteria were detected when the contamination level was higher than 1%. The method proved specific for CMM and did not react with 11 other plant pathogenic bacterial species or subspecies. Further validation of the method for routine detection of CMM in tomato seeds is in progress.

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