SHORT COMMUNICATION

DEVELOPMENT OF A MULTIPLEX PCR ASSAY FOR THE SIMULTANEOUS DETECTION OF CLAVIBACTER MICHIGANENSIS subsp. MICHIGANENSIS, PSEUDOMONAS SYRINGAE pv. TOMATO AND XANTHOMONAS AXONOPODIS pv. VESICATORIA USING PURE CULTURES

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SUMMARY

A multiplex PCR assay for the simultaneous detection of three bacterial seed-borne pathogens of tomato was developed. Published primers: (i) CMM-5-CMM-6 for Clavibacter michiganensis pv. michiganensis; (ii) primer 1-primer 2 for Pseudomonas syringae pv. tomato; (iii) RST2-RST3 for Xanthomonas axonopodis pv. vesicatoria, were used in the assay. Annealing temperatures were determined by gradient PCR individually for each pathogen and primer concentration ratios were investigated. Sensitivity assays were carried out and compared with single PCR. Temperature of 59±1°C was optimal for annealing. Optimal primer concentrations were determined as 0.36 µMol l⁻¹ for C. m. michiganensis, 0.30 µMol l⁻¹ for X. a. vesicatoria, and 0.12 µMol l⁻¹ for P. s. tomato. Sensitivity assays showed that 3 CFU in 50 µl sterile distilled water, derived from pure cultures of C. m. michiganensis, P. s. tomato and X. a. vesicatoria could reliably be detected by multiplex PCR when applied to pure cultures. The detection limit was determined to be ca. 10 times lower than that of single PCR. Multiplex PCR provided less labor and rapid results for the detection of bacterial pathogens of tomato, but the sensitivity of detection was reduced. Thus, the sensitivity of this technique should be assayed prior to its use in place of single PCR.

Key words: Clavibacter michiganensis subsp. michiganensis, Pseudomonas syringae pv. tomato, Xanthomonas axonopodis pv. vesicatoria, tomato, detection, multiplex PCR.

Clavibacter michiganensis subsp. michiganensis, Pseudomonas syringae pv. tomato and Xanthomonas axonopodis pv. vesicatoria are important bacterial pathogens of tomato and cause economic losses worldwide. PCR methods and primers have been developed for each pathogen (Bereswill et al., 1994; Dreier et al., 1994; Leite et al., 1995). Coronatine toxin-producing strains of P. syringae pv. tomato were identified with a 650 bp PCR product, along with other coronatine-producing pathovars of Pseudomonas syringae (Bereswill et al., 1994). Xanthomonas axonopodis pv. vesicatoria, the agent bacterial spot of tomato and pepper, was amplified by three different PCR primer pairs targeted to the hrp gene cluster of the genus Xanthomonas. One of these primer pairs, RST2-RST3 was designed on the hrpB region of Xanthomonas axonopodis pv. vesicatoria strain 75-3 and amplified a 840 bp product (Leite et al., 1994). Specific detection of virulent strains of Clavibacter michiganensis subsp. michiganensis that causes bacterial canker of tomato, was possible, using CMM-5 and CMM-6 primers, from plasmid-borne pat-1 gene which generated a 614 bp product specific to virulent strains (Dreier et al., 1995). The detection limit in this assay, when testing infected plants was ca. 2x10² CFU ml⁻¹.

Multiplex PCR allows the amplification of more than one target region in one PCR reaction mixture and can reduce the time and labor as compared with single PCR (Chamberlain and Chamberlain, 1994). However, the use of multiple primers on multiple templates may result in inefficient or preferential binding of some primers to their templates, while the degree of proximity of annealing temperatures of different primers can negatively affect the outcome of the assay (Elñistro et al., 2000). Nonetheless, multiplex PCR assays have already been employed for detection of a number of pathogens (Glick et al., 2002; Menzel et al., 2002; Bertolini et al., 2003; Hiroyuki and Tsuda, 2005; Özdemir, 2005a).

The aim of this study was the development of a multiplex PCR assay to detect three seed-borne pathogens of tomato, C. michiganensis subsp. michiganensis, P. syringae pv. tomato and X. axonopodis pv. vesicatoria simultaneously in a single PCR tube. A preliminary report of this work has been given previously (Özdemir, 2005b), but this paper now extends the study to the partial optimization of amplification conditions from pure cultures of the pathogens.

For PCR assays, C. michiganensis subsp. michiganensis ICMP 2550 [Type strain (International Collection of Micro-organisms from Plants, Auckland, New Zealand)], P. syringae pv. tomato ICMP 2844 and X. axonopodis pv. vesicatoria ICMP 9592 [75-3, group A
strains, tomato race 1, also classified as X. euvesicatoria, Jones et al., (2004) were routinely grown on King medium B (King et al., 1954), on yeast dextrose carbonate agar [YDCA (Wilson et al., 1967)] and on nutrient broth yeast extract aga [NBYA (Vidaver, 1967)] at 24°C. Cultures were maintained in 30% glycerol at -80°C for long term storage.

PCR primers used in this study, shown in Table 1, had the following sequences: CMM-5: GCGAATAAGCCCATATCAA (19-mer), CMM-6: CGTACCGAGGTCGCTAATA (19-mer) (Dreier et al., 1995); primer 1: GCGGCTCCTCGCACTT (17-mer), primer 2: GGTATGGCCGAGGTGC (17-mer) (Bereswill et al., 1994), RST2: AGGCCTGGAAGGTGCCCTGGA (22-mer), RST3: ATCGCAGCGTACCGCGGCGG (22-mer) (Leite et al., 1994).

Annealing temperatures were determined by gradient PCR. Reaction mixture contained 200 µMol l-1 dNTPs, 1x PCR buffer (100 mMol l-1 Tris-HCl pH 8.8, 500 mMol l-1 KCl, 0.8% Nonidet P40), 1.5 µMol l-1 magnesium chloride, 0.05 units µl-1 Taq polymerase (Fermentas, Lithuania) in 25 µl reaction volume. DNA templates were prepared from 4-day-old cultures grown on their respective agar medium, suspending half loopful from each culture in 200 µl sterile distilled water in eppendorf tubes. Tubes were boiled for 10 min in a thermomixer. For each bacterial culture, three colonies were prepared by boiling for 10 min in an Eppendorf thermomixer. For each bacterial culture, three colonies were suspended in 50 µl sterile distilled water and 10 to 100 fold dilutions were made. An aliquot of 1.25 µl template was used for each pathogen in a final PCR volume of 25 µl. Cycling conditions were 35 cycles at 95°C for 30 sec, 59.4°C for 30 sec and 72°C for 45 sec. Final extension was at 72°C for 5 min. The same amount of primer concentration and reaction mixture was used as in the gradient PCR. The effect of Taq polymerase and Mg2+ concentration was also tested. Under standard magnesium concentration (1.5 mMol l-1), Taq polymerase concentration was increased by 1.6-fold. All PCR experiments were repeated at least twice.

Multiplex PCR requires such an annealing temperature that all templates can be amplified efficiently by their primers. A gradient PCR, with temperatures increasing from 55.2°C to 61.4°C was applied to each template of C. m. michiganensis, P. s. tomato and X. a. vesicatoria. A common annealing temperature for all templates was determined as 59±1°C. In parallel to determination of the annealing temperature, for homogenous amplification of templates, primer concentration ratios of three primer pairs were tested as paired group by PCR. Based on the

<table>
<thead>
<tr>
<th>Primers</th>
<th>Pathogen</th>
<th>Target gene</th>
<th>Fragment length</th>
<th>Tm</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMM-5-CMM-6</td>
<td>Clavibacter michiganensis subsp. michiganensis</td>
<td>pat-1</td>
<td>614 bp</td>
<td>55°C</td>
<td>Dreier et al. (1995)</td>
</tr>
<tr>
<td>primer 1-primer 2</td>
<td>Pseudomonas syringae pv. tomato</td>
<td>efl</td>
<td>650 bp</td>
<td>67°C</td>
<td>Bereswill et al. (1994)</td>
</tr>
<tr>
<td>RST2-RST3</td>
<td>Xanthomonas campestris pv. vesicatoria</td>
<td>hpr</td>
<td>840 bp</td>
<td>62°C</td>
<td>Leite et al. (1994)</td>
</tr>
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</table>
results obtained, it was possible to choose the ratio of 1 (0.12 µMol l\(^{-1}\)) : 2.5 (0.3 µMol l\(^{-1}\)) : 3.6 (0.36 µMol l\(^{-1}\)) for primer 1-primer 2, RST2-RST3 and CMM-5-CMM-6, respectively. After optimization of annealing temperature and primer concentration ratios, the sensitivity threshold of multiplex and single PCR was compared using pure cultures of the pathogens. In both single and multiplex PCR, each template was detected reliably for all three pathogenic bacteria at 3 CFU in 50 µl sterile distilled water. However, lower yields by a 10 factor were obtained in multiplex than in single PCR.

In multiplex PCR, increasing Taq polymerase enzyme concentration 1.6 fold did not have any effect on amplification sensitivity. In addition, increasing the amount of Mg\(^{2+}\) concentration from 1.5 to 3 mMol l\(^{-1}\) did not increase amplification sensitivity. Previously, Özdemir (2005a) had studied simultaneous detection of C. m. subsp. michiganensis and X. a. pv. vesicatoria by multiplex PCR without performing sensitivity assays. Johnson and Walcott (2005) and Fessehaie and Walcott (2005) have shown the detection of Pepino mosaic virus and C. m. subsp. michiganensis by real-time PCR and Acidovorax avenue subsp. citrulli and Didymella bryonieae by multiplex real-time PCR.

In this study, to prepare DNA templates, bacterial colonies were boiled as previously reported (Schaad et al., 1995) and no PCR inhibition was obtained during multiplex and single PCR assays. Further research would be desirable to develop a reliable DNA extraction method of bacterial template from seeds and a sensitive detection method of the three pathogens in the seeds using real-time PCR.

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