Detection of *Acidovorax avenae* subsp. *citrulli* using PCR and MALDI-TOF MS

Hideyuki Kajiwara¹*, Masatoshi Sato² and Akiko Suzuki²

¹ National Institute of Agrobiological Sciences  
² National Center for Seeds and Seedlings

(Received March 29, 2012; Accepted May 6, 2012)

**SUMMARY**

Virulent strains of *Acidovorax avenae* subsp. *citrulli* (*Aac*) and *avenae* (*Aaa*) cause bacterial fruit blotch in cucurbits and bacterial stripe in rice, respectively. Here, we describe a rapid (1 h) method to identify virulent strains of *Aac* using a combination of short PCR and matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). Single-stranded DNA of the PCR products was analyzed by MALDI-TOF MS after digestion by an enzyme and alkali denaturation. The mass spectra differed among the strains as a result of the differences in their nucleotide sequences. This new method allows rapid identification of virulent strains of *Aac*.

**Key words:** *Acidovorax avenae*, matrix assisted laser desorption ionization time-of-flight mass spectrometry, PCR, single nucleotide polymorphism

**INTRODUCTION**

Cucurbits are cultivated worldwide. Bacterial fruit blotch of cucurbits caused by *Acidovorax avenae* subsp. *citrulli* (*Aac*) is a serious threat to production of cucurbit seeds and fruit. *Aac* is seed-transmitted and therefore, is spread through transportation of infected seed. *Acidovorax avenae* subsp. *avenae* (*Aaa*) causes several important plant diseases including bacterial stripes of rice, bacterial stalks rot of corn, bacterial leaf blight of oats, and red stripes of sugarcane. There are several subspecies within *A. avenae*, including *avenae*, *cattleyae*, and *citrulli*. Their evolutionary relationship has been analyzed using 16S rRNA sequences. *Aac* strains have also been analyzed using DNA fingerprinting by pulse-field gel electrophoresis and repetitive extragenic palindromic PCR. Cluster analyses based on the fingerprinting profiles divided the *Aac* strains into two groups. Several approaches have been used to detect *Aac* and *Aaa* strains including BIO-PCR, which combines agar plating of *Aac* and real-time PCR assays using enriched bacteria. Immunomagnetic separation and PCR-based assays have also been used to detect *Aac*, and recently, a simultaneous detection method was developed. Intraspecific variation was also analyzed using PCR, DNA fingerprinting, and whole cell fatty acid analysis. Those investigations were aimed at *Aac* disease control; however, species-specific detection is essential because some *Aac* strains are not virulent in some Cucurbits. Although there have been several attempts to identify *Aac* and *Aaa* strains, especially virulent strains, the existing methods are very time-consuming.

Recently, we developed a new method to analyze DNA combining short PCR and matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). This method was originally developed for rapid detection of a specific transgene in transgenic plants. In the method, the amplicon obtained by short PCR was digested by a restriction endonuclease or urasil-DNA glycosylase (UDG). The digested fragment was collected on streptavidin (SA)-coated magnetic beads by capturing the biotin attached to one of the PCR primers. The single-stranded DNA (ssDNA) was alkali-denatured and then analyzed by MALDI-TOF MS. Theoretically, the method identifies single nucleotide polymorphisms (SNP) on the basis of differences in mass resulting from differences in nucleotide sequences.

Here, we report a new method combining short PCR and MALDI-TOF MS analysis for identification of *Aac* strains based on intraspecific variations in *HrpG-HrpX* genes in the *Aac* genome.

**MATERIALS AND METHODS**

**Materials**

General laboratory chemicals were purchased from Wako Pure Chemical Industries (Japan) or Nacalai Tesque.
(Japan) unless otherwise stated. Three strains of *A. avenae* subsp. *citrulli* and *avenae* designated as *Aac*1, *Aaa*5, and *Aaa*10 were used in this study (Table 1). Strain *Aac*1 is a virulent strain isolated in Yokohama, Japan (Strain number Aac9801). Strains *Aaa*5 and *Aaa*10 are virulent strains isolated in Japan (National Institute of Agrobiological Sciences, Japan). All *A. avenae* strains were grown on nutrient broth (YP) for 16 h at 36°C[11]. Cells were collected by centrifugation and stored at ~20°C until use.

**PCR and MALDI-TOF MS analysis**

For each strain, a specific DNA region was amplified using a combination of one unlabeled primer and one primer that was biotinylated at the 5’-end. PCR primers were as follows: *Aac*125L (5’-AAG ACA TTC ACA ATT TTT GCA C-3’), *Aac*181RB (biotin-TCT GTC GCC ACT CAC GCA C-3’), *Aac*348LB (biotin-CGG ATA CCA ATA ACT TTC CTA TA-3’), *Aac*405R (5’-ATG CGG GCC ATG AGC GCC GT-3’), *Aac*419LB (biotin-GCT GCC TTC GGG TTC GGC GT-3’), and *Aac*480R (5’-CTC ACC TAG GTT TTC CAA ATC CTC AAA TTT-3’). Oligonucleotides were synthesized by Hokkaido System Science Co., Ltd., Japan. PCR was performed in a 20 μl reaction volume using PCR Master Mix (Promega, USA) or *Tfi* DNA polymerase (Invitrogen, USA). The reaction was denatured for 1 min at 94°C, then thermocycled 30 times as follows: 2 s at 94°C, 2 s at 60°C, and 2 s at 72°C (2720 Thermal Cycler, Applied Biosystems, USA). dUTP was substituted for dTTP when UDG (New England Biolabs, USA) was going to be used to cleave the PCR products. Electrophoresis was performed on 20% acrylamide gels in Tris-borate buffer[12].

An aliquot (5 μl) of PCR product was incubated with a restriction enzyme or with UDG for 10 min according to the manufacturer’s instructions (New England Biolabs). Digested DNA fragments were bound to 2 μl SA-coated magnetic beads (Takara Bio Inc., Japan) and then collected by washing with water. Then, 50 μl denaturing solution containing 0.1 M NaCl and 0.1 M NaOH was added and the mixture was incubated for 5 min. The beads were washed twice with water, then mixed with 50 μl 25% ammonia solution and kept at 65°C for 10 min. The solution was collected and lyophilized using a vacuum concentrator. ssDNA was dissolved in 0.15 M diammonium hydrogen citrate and spotted onto the target (Anchor Chip, Bruker Daltonics, Germany) and the same volume of 100 mg/ml 2’,4’,6’-trihydroxyacetophenone in 50% acetonitrile was added. Spectra were recorded by a MALDI-TOF MS (Ultraflex, Bruker Daltonics) in the linear positive ion mode[10].

**RESULTS AND DISCUSSION**

In these analyses, one of the primers had to be biotinylated at the 5’-end for collection by SA-coated magnetic beads after PCR (Table 1). In addition, a new restriction site and/or a cleavage site had to be created in the amplified DNA by PCR to show differences among the *A. avenae* strains. After amplification, a restriction enzyme or UDG was added to cleave the amplicons at a specific sequence (Table 1). DNA fragments were collected by binding of the biotin located at the 5’-end to SA-coated magnetic beads. Double-stranded DNA was denatured under alkaline conditions to generate ssDNA, and ammonia solution was added to dissociate the bond between biotin and SA. ssDNA was collected by lyophilization for subsequent analysis by MALDI-TOF MS. Therefore, the selection of amplification region and the design of primers are most important in this method.

The following predictions could be made on the basis of the MALDI-TOF MS spectra: using the primer set

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Table 1. Strains, *Aac*1, *Aaa*5, and *Aaa*10 used in this study and the nucleotide sequences. Primer sequences for PCR and the amplicon were showed. The region for the PCR amplification was selected in the specific genes (*HrpG-HrpX*) with the help of the software Primer3 (http://primer3.sourceforge.net/). Addition of A residue was ignored.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Primers and amplified sequence</th>
<th>Restriction enzyme and UDG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Primer</td>
<td><em>Aac</em>125L</td>
</tr>
<tr>
<td><em>Aac</em>1</td>
<td>5’-AAGACATTCACAATTTTTGCA</td>
<td>T</td>
</tr>
<tr>
<td></td>
<td>CTACATGGCAAATTTGAGGATTTG</td>
<td>CC</td>
</tr>
<tr>
<td><em>Aaa</em>5</td>
<td>5’-GG-T</td>
<td>T</td>
</tr>
<tr>
<td><em>Aaa</em>10</td>
<td>5’-GG-T</td>
<td>T</td>
</tr>
<tr>
<td><em>Aac</em>1</td>
<td>5’-CCGATACCAATTAATTTTTGCA</td>
<td>T</td>
</tr>
<tr>
<td></td>
<td>CTACATGGCAAATTTGAGGATTTG</td>
<td>CC</td>
</tr>
<tr>
<td><em>Aaa</em>5</td>
<td>5’-CCGATACCAATTAATTTTTGCA</td>
<td>T</td>
</tr>
<tr>
<td><em>Aaa</em>10</td>
<td>5’-CCGATACCAATTAATTTTTGCA</td>
<td>T</td>
</tr>
</tbody>
</table>

* One of the primer sequences (underlined and opposite of broken line) had to be biotinylated at the 5’-end (*Aac*181RB, *Aac*348LB, and *Aac*419LB).
Aac125L-Aac181RB, the amplicon would be 57 bp (Table 1). Therefore, the ssDNA fragment obtained by digestion with *Mnl* treatments and subsequent alkali denaturation would be 26 bases. The amplicon from strain *Aac1* would be digested by *Mnl*, whereas those from strains *Aaa5* and *Aaa10* would not be cleaved by *Mnl* because of no amplicon (Table 1). The primer set *Aac348LB-Aac405R* contained biotin on the opposite primer compared with the primer set *Aac125LB-Aac181R*. Thus, the resulting amplified DNA would be also 57 bp. In this case, *BceAI* could cleave the amplicon, yielding a 27 base biotinylated ssDNA after enzymatic digestion, alkali denaturation, and collection on SA-coated magnetic beads. *BceAI* would cut the amplicon from strains *Aac1* and *Aaa5*, but no amplicon from them.

In same manner, the primer sets *Aac348LB-Aac405R* and *Aac419LB-Aac480R*, would yield amplicons of 59, and 63 bp, respectively. The resulting ssDNA obtained after enzymatic reactions and alkali denaturation would be 27 and 18 bases in length, respectively. Fragmentations of amplicons by enzymes are summarized in Table 1.

The primer sets used here yielded amplicons that could be visualized as bands after electrophoresis (data not shown). Although the PCR products derived from the three strains (*Aac1*, *Aaa5*, and *Aaa10*) produced bands at the same position on an acrylamide gel, they had different nucleotide compositions and length. Therefore, conventional electrophoresis could not show differences among the amplicons. Single-strand conformation polymorphism (SSCP) analysis could be used to detect differences among the amplicons; however, this method is time-consuming and it cannot be used to analyze many samples simultaneously. For these reasons, it is difficult to use SSCP for commercial purposes. If the target SNP was clear, direct DNA sequencing might be faster than SSCP analysis.

Using the primer set *Aac125L-Aac181RB*, the amplicon obtained by PCR was 57 bp. If the resulting amplicon is very short, the primer extension time can be shortened to 2 s, resulting in a total PCR time of less than 40 min. If better PCR apparatus is used to amplify the target sequence, the total reaction time would be less than 40 min because most of the reaction time allows for changes in temperature. Fast PCR could be used for this procedure, and the total amplification time could be shortened to less than 20 min for DNA samples with higher annealing temperatures (data not shown).

The spectra of products obtained using primer set *Aac125L-Aac181RB* are shown in Fig. 1A. In this case, the ssDNA fragment obtained after enzymatic reactions would

![Fig. 1. MALDI-TOF MS analysis of amplicons.](image-url)

A. Peak at m/z 8277 (arrow) represents ssDNA fragment derived from primer set *Aac125L*-*Aac181RB* after digestion by *Mnl* and alkali denaturation. B. Peak at m/z 8581 (arrow) represents ssDNA fragment derived from primer set *Aac348LB-Aac405R* after digestion by *BceAI* and alkali denaturation. C. Peak at m/z 7534 (arrow) represents DNA fragment derived from primer set *Aac348B-Aac405R* after digestion by UDG and alkali denaturation. D. Peak at m/z 5579 (arrow) represents DNA fragment derived from primer set *Aac419LB*-*Aac480R* after digestion by *Mnl* and alkali denaturation. *: Peak derived from unreacted biotinylated primer.
be longer than the seven bases of the unreacted biotinylated primer Aac181RB. As expected, there was no peak in strains Aaa5 and Aaa10. The non-biotinylated primer would not be collected by the SA-coated magnetic beads. In the MALDI-TOF MS analysis, the ionization ratio of molecules with higher mass is lower than that of molecules with lower mass. Thus, the peak associated with products that were seven bases longer than the biotinylated primer was very small. Although the peak derived from PCR and enzymatic digestions was able to be detected by MALDI-TOF MS analysis, we concluded that the primer design should be reconsidered for clearer identification of A. avenae strains in this case.

In the case of primer set Aac348LB-Aac405R (Fig. 1B), we observed a new fragment that was three bases longer than the biotinylated primer Aac125LB. Only strain Aac1 showed a peak in the MALDI-TOF MS analysis. There was no peak in strains Aaa5 and Aaa10. Therefore, strains Aac1, Aaa5, and Aaa10 could be distinguished by the peaks obtained from primer set Aac125LB-Aac181R and primer set Aac125L-Aac181RB.

In the case of the primer set Aac348LB-Aac405R (Fig. 1C), UDG was used to cleave the DNA products obtained by PCR. dUTP was used for PCR amplification in the UDG treatment. UDG cut the DNA strand at nucleotide U, which replaced nucleotide T during the PCR amplification. Strain Aac1 showed a new peak close to the peak derived from the biotinylated primer Aac419LB, while the other strains did not show this peak because of no amplicon. The restriction enzyme MnlI was used to analyze strains Aac1, Aaa5, and Aaa10 (Fig. 1D). The MnlI recognition site was located apart from the cleavage site, and so the resulting product was six bases shorter than the biotinylated primer Aac419LB. The peak height was higher than those of the peaks obtained using the other primer sets (shown in Fig. 1A–C). This is because of the higher ionization ratio for molecules with a lower mass in the MALDI-TOF MS analysis. Theoretically, strain Aaa5 should not have shown a peak because there was no amplicon (Table 1). The reason for the small peak in strain Aac5 remains unclear; however, there may have been some contamination from strain Aac1, or there may be minor variations in strain Aaa5.

The GOOD assay is a similar approach for the analysis of SNPs, which involves nested PCR and special primers coupled with MALDI-TOF MS to detect SNPs. This assay also combines PCR and MALDI-TOF MS\(^1\). Two separate PCR amplifications are required for the GOOD assay, and therefore, the reaction time is longer and the cost of DNA polymerase is higher than those of the new method. The method reported here requires only one PCR amplification. Since the method requires amplification of only a very narrow region of the target gene in the genome, the PCR amplification time can be shortened. When the method reported here was used to detect a transgenic gene in rice seeds, crude extracts from rice grains could be used for the PCR template to produce 50 to 60-bp amplicons\(^1\). In the GOOD assay, the first PCR amplifies a relatively wide region before the second PCR amplification using dideoxynucleotides, and purified genomic DNA is required as template for the first PCR. If the GOOD assay was used to detect virulent Aac strains, a pure genomic DNA template for each strain would be required for the first PCR.

It is possible to detect Aac strains in 1 h using this combined PCR and MALDI-TOF MS technique. The advantages of this procedure over the conventional methods based on PCR and electrophoresis analysis are the short reaction times, including extraction of template DNA, and the low cost of consumables (DNA polymerase, primers, and gels). The entire procedure, from template extraction for PCR to MALDI-TOF MS analysis, can be automated because there is no requirement for manual electrophoresis. Construction of the automated method is underway in our laboratory. Here, we applied the method to identify three A. avenae strains\(^1\) and related proteobacteria can be obtained, virulent Aac strains can be identified in 1 h using this method. Thus, in the future, it would be possible to detect bacterial fruit blotch of cucurbits at quarantine stations, even before symptoms are visible.

REFERENCES


