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rDNA-ITS sequence analysis of pathogens of cucumber downy mildew and cucumber powdery mildew

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Abstract To determine the pathogens of cucumber downy mildew and cucumber powdery mildew by molecular marker, we amplified and sequenced the rDNA-ITS region of the pathogens of cucumber downy mildew and cucumber powdery mildew collected from the Shanghai region. The intra-/interspecific sequence difference was analyzed by rDNA-ITS sequence. The results show that the length of rDNA-ITS1 and rDNA-ITS2 of cucumber downy mildew's pathogen was 141 bp and 406 bp, respectively, with GC contents of 41.13% in ITS1 and 46.8% (Minhang and Jinshan District, sm1 and sm2) or 46.55% (Pudong District, sm3) in ITS2. The rDNA-ITS sequence was intraspecific conservation. The interspecific difference was related with their kin relationship. The pathogen of cucumber downy mildew was identified as *Pseudoperonospora cubensis* by molecular marker. The length of rDNA-ITS1 and rDNA-ITS2 of cucumber powdery mildew's pathogen was 136 bp and 89 bp, respectively, with GC contents being 59.56% and 66.29%, and rDNA-ITS sequence being highly conservative in this study that was the same as *Sphaerotheca cucurbitae*. But the sequence difference between the strains in the Shanghai region in this study with *S. fuliginea* was 4.5%, which was identified by morphology. It is suggested that the pathogen of cucumber powdery mildew should be further clarified and determined.

Keywords cucumber downy mildew's pathogen, cucumber powdery mildew's pathogen, rDNA-ITS

1 Introduction

Downy mildew and powdery mildew are two important diseases in cucumber, and have a wide epidemic range.

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The cucumber downy mildew is commonly called “Pao-Ma-Gan”, “Hei-Mao” or “Wen-Bing” in Chinese. The total cucumber leaves would dry, rot and die rapidly when infected with the pathogenic fungi, which would decrease the cucumber quality and yield dramatically. The pathogen of the cucumber downy mildew was *Pseudoperonospora cubensis* (Wei, 1979). The cucumber powdery mildew is commonly called “Bai-Mao” in Chinese. When the cucumber plant was infected with powdery mildew's pathogen, the mycelium would grow in the leaves, then form white round colonies and even cover the whole leaves. They are harmful not only to the leaves but also to the stems. The recorded pathogens for cucumber powdery mildew were different according to previous studies. According to “Flora Fungorum Sinicorum (Chen et al., 1987)”, it was *Sphaerotheca cucurbitae* or *Erysiphe cucurbitacearum*. Some other researchers reported that it was *E. cichoracearum* or *S. fuliginea* (Qiu, 1979). Therefore, the clarification of the pathogens of the above two diseases is necessary.

The ribosomal DNA internal transcribed spacer (rDNA-ITS) show its suitability for diagnosing and phylogenetic relationship among cryptic fungi species because of its considerably evolutionary rate (Yu et al., 2000; Voglmayr, 2003). But no molecular information about the pathogens of cucumber downy mildew and cucumber powdery mildew has been reported in China. In this paper, we sequenced and analyzed the rDNA-ITS region of the above two pathogens to clarify the taxonomic status by molecular identity.

2 Materials and methods

2.1 Materials

2.1.1 Source of pathogens

The fungi pathogens of the cucumber downy mildew were collected from cucumber planting fields of the Minhang District (sm1), the Jinshan District (sm2) and the Pudong District (sm3) in Shanghai, China. The fungi pathogen of

the cucumber powdery mildew was collected from the Minhang District (bf1), Pudong District (bf2) and Songjiang District (bf3) also in Shanghai, China. The two kinds of pathogenic fungi were isolated from the infected plants and identified as *Pseudoperonospora cubensis* and *Sphaerotheca fuliginea* according to their symptoms and pathological features by the researchers from the College of Agriculture and Biology, Shanghai Jiaotong University.

2.1.2 Instruments and reagents

The major instruments and reagents used in this study were: Thermostatic Water-bath (The 5th Shanghai Medical Equipment Factory), Peltier Thermal Cycler (PTC-100, BIO-RAD), *Taq* Polymerase (Shanghai Sangon), 10 × PCR buffer (Shanghai Sangon), dNTP (Shanghai Sangon) and Gel Extraction Kit (Qiagen).

2.2 Genomic DNA extraction

Genomic DNA was extracted using the modified protocol of Yi et al. (2002). The infected leaves were flipped softly by hand and then the spores and mycelium were dropped in a buffer solution on the slide. They were crushed with needles under an anatomical lens and transferred to a centrifuge tube and thereafter centrifuged at 12000 r·min⁻¹ for 5 min. Then, 10 μL Tween-20 was added and was incubated at 60°C for 3 h. Genomic DNA from the supernatant was preserved and stored at -20°C until usage.

2.3 rDNA-ITS amplification and sequencing

2.3.1 PCR primers

Nested PCR was carried out to amplify rDNA-ITS region of the two pathogens. The outer primers were the same referred by the universal primers of fungi (Motoaki and Takashi, 1995; Tetsuya and Susumu, 1996; White et al., 1990): ITS5 (5'-GGA AGT AAA AGT CGT AAC AAG G-3') and P3 (5'-GCC GCT TCA CTC GCC GTT AC-3'). The inner primers of the cucumber downy mildew's pathogen were designed based on *P. cubensis* sequence (Voglmayr, 2003), with psm1 (5'-AAC TTT CCA CGT GAA CTG TAT-3') and psm2 (5'-TGA GAT GCC GCG CGA CCG AAG-3'). The cucumber powdery mildew's inner primers were designed according to *S. fuliginea* (AB026144) (Takamatsu et al., 2000), with pbf1 (5'-GTA GGT GAA CCT GCG GAA GGA T-3') and pbf3 (5'-CGC GAG ATA CAT GAC TAC GC-3'). All the primers were synthesized by the Shanghai Sangon Company.

2.3.2 Amplification and sequencing

The PCR reaction mixture of 50 μL contained 1.5 U *Taq* DNA polymerase, 10 mmol·L⁻¹ Tris (pH 8.3),

50 mmol·L⁻¹ KCl, 1.5 mmol·L⁻¹ MgCl₂, 0.1 mg·mL⁻¹ BSA, 0.1 mmol·L⁻¹ of dNTPs, 0.1 μmol·L⁻¹ of each primer and 2 μL of genomic DNA. The cycling program included pre-denaturing at 94°C for 90 s and 30 cycles at 94°C for 30 s, at 50°C for 30 s, and at 72°C for 30 s. PCR products were checked on 2% agarose gels (0.5 μg·mL⁻¹ ethidium bromide) and purified by kit's protocol. The rDNA-ITS region was sequenced by ABI Prism BigDye terminator cycle sequencing protocol on ABI 377 (Shanghai UG).

2.4 Sequence analysis

Sequences were aligned by Clustal X software (Version 1.8) and manual adjustment as necessary. The intra- and inter-species variability were calculated by the Bioedit program (Version 5.0.9). The other sequences of the cryptic species were obtained from GenBank.

3 Results

3.1 rDNA-ITS characters of cucumber downy mildew's pathogen

The 709-bp length of rDNA-ITS region was amplified and specially sequenced from three Shanghai strains (sm1, sm2 and sm3) of the cucumber downy mildew's pathogen. It could be divided into three parts: partial ITS1 fragment (1–141 bp), total 5.8 S coding regions (142–303 bp) and partial ITS2 fragment (304–709 bp) referred by the reported sequences (Voglmayr, 2003). The length of ITS1 and ITS2 was 141 bp and 406 bp, respectively, GC contents were 41.13% in ITS1 and 46.18% (sm1 and sm2) or 46.55% (sm3) in ITS2 with cucumber downy mildew's pathogen. The GenBank accession numbers of the above sequences were DQ025515 (sm1), DQ025516 (sm2) and DQ025517 (sm3). After alignment analysis we found that the sequences of sm1 and sm2 strains were completely identical and there was only single base transition (G → A) on site 640 in ITS2 fragment as comparing sm3 with sm1/sm2 strains.

In this study, the sequences of cucumber downy mildew's pathogen of the Shanghai strains were aligned with two other strains and five cryptic species. It was shown that sm1/sm2 stains were identical with Austria HV222 strain (AY198306) of *P. cubensis* and there were two sites of single base conversion at 465 (C → A) and 652 (A → C) in ITS2 fragment as compared with the Nanjing strain (AY744946). The results suggested that the pathogen species of cucumber downy mildew in this study is *P. cubensis* according to molecular identity. Comparing it with cryptic species, we found that there were 3 variations between sm1 strain and *P. humuli* (AY198305), with A → C on site 380, A → G on 563 and A → G on 640,

respectively, and 22 variations between sm1 strain and *P. urticae* (AY198307) and the divergence rates were 7.1%, 7.5% and 7.7%, respectively, compared with *P. stigmatiicola* (AY198295), *P. tranzscheliana* (AY198294) and *P. radii* (AY198296). The variance bases were almost located in ITS1 and ITS2 fragments. The 5.8 S coding region was relatively conserved.

3.2 rDNA-ITS characters of cucumber powdery mildew's pathogen

The 379-bp length of rDNA-ITS region was amplified and specially sequenced from three Shanghai strains (bf1, bf2 and bf3) of cucumber powdery mildew's pathogen. It could be divided into three parts referred by Takamatsu et al. (2000): partial ITS1 fragment (1–136 bp), total 5.8 S coding region (137–290 bp) and partial ITS2 fragment (291–379 bp). The length of ITS1 and ITS2 fragments was 136 bp and 89 bp, with GC content of 59.56% and 66.29%, respectively. The GenBank accession numbers were DQ025518 (bf1), DQ025519 (bf2) and DQ025520 (bf3). The three sequences in our study were all identical. We sent them to do GenBank-BLAST and found that they were identical with *S. cucurbita* MUMH65 strain (AB026146) from Japan, but differed with *S. fuliginea* (AB026144) at 4.5%. The results suggested that the molecular identity of cucumber powdery mildew's pathogen is confused.

In this study, rDNA-ITS sequences of the cucumber powdery mildew's pathogen Shanghai strains were aligned with the other strains and its cryptic species (Takamatsu et al., 2000; Hirata et al., 2000; Takamatsu et al., 1998; Kiss et al., 2001). The results show that the Shanghai strains were identical with the *S. cucurbita* MUMH65 strain (AB026146) from Japan, and there were 2, 4, 17 and 34 base variations with *S. balsaminae* (AB040318), *S. fusca* (AB026148), *S. fuliginea* (AB026144) and with *S. aphanis* (AB000938), respectively, and 73 bases variation with *Erysiphe cichoracearum* (AF229018). The variance base sites were almost located in the ITS1 and ITS2 fragments, and the 5.8 S coding region was relatively conservative.

4 Discussion

rDNA-ITS sequence of *P. cubensis* (the pathogen species of cucumber downy mildew) was almost the same among the reported strains, including the Nanjing strain, Austria strain and Shanghai strain in our study, which proved that the rDNA-ITS region had intraspecific conservation. The divergence rate was positively related with their kin relationship among the cryptic species, which included two species of the same genus *Pseudoperonospora* and three species of genus *Peronospora*. In our study it was further

confirmed that rDNA-ITS sequence was a suitable molecular marker for identifying the cryptic fungi species (Yu et al., 2000). We also found that the level on inter-strain rDNA-ITS sequence variation of the same species was from zero to great with large gaps among them during alignment analysis and the sequences were all from the GenBank database. It might be caused by wrong sample identification or something else. Alignment analysis must be carefully done. The results also show that the divergence rate between *P. cubensis* and *P. humuli* was similar to the intraspecific level, which suggested that the taxonomic status on the two species should be further studied in future.

In our study, the rDNA-ITS sequence of cucumber powdery mildew's pathogen was identical with *S. cucurbita* MUMH65 strain from Japan (Takamatsu et al., 2000), but greatly different with *E. cichoracearum* and *S. fuliginea* (Kiss et al., 2001). Otherwise, the rDNA-ITS sequence of *E. cucurbitacearum*, which was also considered as the pathogen of cucumber powdery mildew, has not been submitted. Therefore, it is still difficult to make comparison. It was thus evident that the species identification of the cucumber powdery mildew's pathogen was still confused. The samples in this study were distinguished as *S. fuliginea* by symptom and morphology. It was not the same as the recorded species (*S. cucurbitae* and *E. cucurbitacearum*) by "Flora Fungorum Sinicorum" and there was also a great divergence in rDNA-ITS sequences of *S. fuliginea*. These variations suggested that the pathogen of cucumber powdery mildew in Shanghai should be further clarified.

The pathogens of cucumber downy mildew and cucumber powdery mildew are all obligate parasites and cannot be cultured *in vitro*. They cannot live without a plant host. Pure pathogenic fungi were difficult to isolate from the infected leaves during the genomic DNA extraction. In order to avoid the unwanted hosts or other plant genomic DNA mixture which may result in a PCR amplification failure, we flipped the infected leaves to collect the pure pathogen samples and then removed the mixtures under an anatomical lens. The specificity was improved and the impure template was also avoided by nested PCR amplification.

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