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Composition of the endophytic filamentous fungi isolated from the tea plant *Camellia sinensis*

Received: 30 November 2005 / Accepted: 24 January 2006 / Published online: 14 April 2006
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Abstract It has been found by ribosomal DNA analysis that the endophytic filamentous fungi isolated from the tea plant *Camellia sinensis* (Theaceae) are composed of six groups; one *Fusarium* sp., one *Penicillium* sp., two *Schizophyllum* sp., and two *Diaporthe* sp.

Keywords Endophyte · Filamentous fungi · *Camellia sinensis* · rDNA · *Fusarium* sp. · *Penicillium* sp. · *Schizophyllum* sp. · *Diaporthe* sp.

Introduction

It is known that there are several symbiotic relationships in nature. We are, among them, paying attention to that between plants and microorganisms, which are divided into endophytes [1] living inside of a plant and epiphytes living at surface of a plant. To know the composition of endophytes is useful since they create the distinctiveness of each plant.

We previously reported that 35 filamentous endophytic fungi were obtained from the young stems of *Camellia sinensis* (L.) O.K. collected in West Java, Indonesia [2,3]. In this paper, we deal with classification in a genus level of the endophytic filamentous fungi by ribosomal DNA analysis.

Materials and methods

Plant material and isolation procedure of the endophytic filamentous fungi (Code No. PCS-1–PCS-35) were car-

ried out through the same methods as described in our previous papers [2–4].

DNA extraction

Each endophytic fungus was ground down in lysis buffer (400 µl, 50 mM Tris-HCl, 50 mM EDTA-2Na, 3% SDS, 1% mercaptoethanol; pH 7.2) containing alumina powder. The suspended liquid was heated at 60°C for 1 h. After cooling, chloroform (200 µl) and TE-saturated phenol (200 µl) were added to the mixture and then submitted to centrifugation at 15,000 rpm for 5 min. The upper phase was collected and treated with 3 M aqueous sodium acetate (10 µl) and isopropanol (200 µl). The whole mixture was submitted to centrifugation at 15,000 rpm for 15 min. The precipitates were collected and treated with 70% EtOH. The whole mixture was again submitted to centrifugation, and the precipitates were collected and dried under reduced pressure, then treated with RNase (0.5 µg, Sigma, MO, USA) in TE buffer solution (10 µl, TE buffer: 10 mM Tris-HCl, 1 mM EDTA 2Na; pH 8.0) at room temperature for 10 min. Finally, the removal of RNA was confirmed by electrophoresis.

RAPD analysis

The extracted DNAs from the endophytic filamentous fungi (PCS-1–PCS-35) were performed in a mixture of 50 ng of the DNA template, 5 µl of a primer [either primer a (5'-GTAGACCCGT-3') or primer b (5'-CCCGTCAGCA-3')], Ready-To-Go RAPD Analysis Beads (Amersham Biosciences Corp. NJ, USA) and sterile distilled water to a final volume of 25 µl. The whole mixture was placed in a thermal cycling process programmed for 5 min at 95°C followed by 45 cycles of 1 min at 95°C, 1 min at 36°C and 2 min at 72°C. The PCR products were then loaded on 2.3% agarose gel at 50 V and 140 mA for 240 min, and then stained in

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ethidium bromide for 20 min. The electrophoresis patterns were visualized under UV light as shown in Fig. 1 to classify them into six groups from fungi A–F.

PCR amplification

PCR amplification of 18S rDNA and ITS1-5.8S rDNA-ITS2 regions was performed in a reaction mixture of 50 mM Tris-HCl buffer (pH 8.0), 100 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 50% glycerol, 1.0% Triton X-100, 2.4 mM MgCl₂, 100 mM of each dNTP (Takara), 0.15 μ M of primers described in our previous paper [3] with about 1 ng of genomic DNA and 1 unit of the *Taq* DNA Polymerase (Promega, UK). The reaction mixture was placed in a thermal cycling process programmed for 0.5 min at 95°C, followed by 30 cycles of 1 min at 95°C, 0.5 min at 55°C, 2 min at 72°C, and a final extension at 72°C for 10 min. The PCR product was checked by electrophoresis in 1% agarose gel with ethidium bromide 0.5 mg/ml. The presence of a single bright band in each lane confirmed successful amplification. The PCR product was suspended in 95% EtOH and placed at –80°C for 27 min, and then the whole was submitted to centrifugation at 15,000 rpm for 15 min. After removal of the supernatant, the residue was resuspended in 250 μ l of 70% EtOH and centrifuged at 15,000 rpm for 5 min, and the precipitates were collected and dried under reduced pressure to obtain a DNA template.

DNA sequencing and phylogenetic analyses

Amplification was performed in a reaction mixture of 1 unit of BigDye Terminator Cycle Sequencing Ready

Reaction Kit V2.0 (ABI), 0.3 μ M of primers described in our previous paper [3] and 15 ng of the DNA template. The reaction mixture was placed in a thermal cycling process programmed for 1 min at 96°C, followed by 25 cycles of 0.5 min at 96°C, 0.25 min at 55°C, 4 min at 60°C, and a final extension at 60°C for 10 min. One microliter of 6 M aqueous ammonium acetate and 30 μ l of 99.5% EtOH were added to the reaction mixture and the whole was shaken briefly and then allowed to stand in an ice-bath for 15 min. The mixture was centrifuged at 14,000 rpm for 15 min. The supernatant was removed and the residue was suspended in 200 μ l of 70% EtOH and centrifuged at 14,000 rpm for 10 min. The resulting precipitates were collected and dried under reduced pressure to afford the cloned DNA fragments. Those fragments were analyzed using a DNA sequencer. Sequence alignment, a similarity matrix, and a neighbor-joining phylogenetic tree were created on a Macintosh personal computer using the DNASTAR program (DNASTAR Inc, Madison, WI, USA) and the Clustal V software package [5,6].

Results and discussion

The endophytic filamentous fungi isolated from the tea plant *C. sinensis* were classified into six species (fungi A–F) by RAPD analysis (Fig. 1).

As to fungus A, the generic name was supposed to be *Gibberella* or *Fusarium*, since the base sequence of the 18S rDNA region showed 99.7% and 99.6% similarities with those of *Gibberella fujikuroi* (DDBJ/EMBL/GenBank, accession No. AR168094) and *Fusarium oxysporum* (AB110910). The genus *Fusarium* is an anamorph stage of the genus *Gibberella* [7,8].

Fig. 1 RAPD band patterns of the endophytic filamentous fungi with primers a (5'-GTAGACCCGT-3') and b (5'-CCCGTCAGCA-3') on 2.3% agarose gel

Fig. 2 Phylogenetic relationship for ITS1, 5.8S rDNA and ITS2 regions between the endophytic fungus A and published data. Numbers above branches are bootstrap values

Next we analyzed the ITS1, 5.8S rDNA and ITS2 regions of fungus A, and examined the phylogenetic relationship with published data. As seen in Fig. 2, fungus A was more close to *Fusarium* sp. than *Gibberella* sp. In a fact, the similarity with those of *Fusarium incarnatum* (AY633745) was 95.7% for the ITS1 region, 100% for the 5.8S rDNA region, and 99.3% for the ITS2 region respectively.

From these findings, it has been clarified that the endophytic fungus A (registered as AB245442) belongs to the genus *Fusarium*.

The generic name of fungus B was presumed to be *Eupenicillium* or *Penicillium* based on the phylogenetic relationship for 18S rDNA region as shown in Fig. 3 and comparison of the 18S rDNA with available sequences in the database, namely the base sequence which showed 95.2 and 94.8% similarities with those of *Eupenicillium javanicum* (U21298) and *Penicillium namyslowskii* (AB028190) respectively. Finally, it was concluded that fungus B (registered as AB245443) is *Penicillium* sp., which is an anamorph stage of *Eupenicillium* sp., [7,8] since no sexual reproductive organs could be observed by light microscopy.

Fig. 3 Phylogenetic relationship for 18S rDNA between the endophytic fungus B and published data. Numbers above branches are bootstrap values

Fig. 4 Phylogenetic relationship for the ITS1, 5.8S rDNA and ITS2 regions between the endophytic fungi C and D and published data. Numbers above branches are bootstrap values

Fig. 5 Comparison of the 18S rDNA region of endophytic fungi C and D with those of *S. commune*

The RAPD fragment patterns of fungi C and D were mutually different as shown in Fig. 1; however, the base sequences for the 18S rDNA, ITS1, 5.8S rDNA and ITS2 regions were completely identical. The length was 2145, 153, 152 and 243 bp for the 18S rDNA, ITS1, 5.8S rDNA and ITS2 regions respectively.

The base sequence in the 18S rDNA region of the endophytic fungi C and D did not show high similarities with those of any fungi in the database. Based on the phylogenetic consideration for the ITS1, 5.8S rDNA and ITS2 regions shown in Fig. 4, fungi C and D were assessed to be *Schizophyllum* sp. In particular, *Schizophyllum commune* (AF280757) gave 100% for ITS1, 99.3% for 5.8S rDNA and 99.6% similarity for ITS2 regions with fungi C and D.

On the other hand, the base sequence for 18S rDNA of fungi C and D was quite unique and did not show a high percentage similarity with those of any fungi in the database. In the 18S rDNA region, the 398 bp fragment was inserted between 5'-side 1124 bp (98.8% similarity) and 3'-side 623 bp (99.2% similarity) in that of *S. commune* as shown in Fig. 5.

These findings indicated that the endophytic fungi C (registered as AB245444) and D (registered as

AB245445) might belong to the genus *Schizophyllum* and be new species.

With regard to fungi E (registered as AB245446) and F (registered as AB245447), we already reported these to be quite closely-related species to *Diaporthe phaseolorum* var. *sojae* (IFO6709) and *D. phaseolorum* strain sw-93-13 (AF001018) respectively [3, 9]

As a conclusion, it has been clarified that the endophytic filamentous fungi isolated from the tea plant *C. sinensis* consist of one *Fusarium* sp., one *Penicillium* sp., two *Schizophyllum* sp. and two *Diaporthe* sp.

Acknowledgements We thank Dr. Partomuan Simanjuntak for collection of the plant material, Mrs. Admarita Agusta for technical assistance, and the National Collection of Industrial, Food and Marine Bacteria (NCIMB) Japan for morphological observation of the endophytic filamentous fungus B *Penicillium* sp. This work was supported by the "High-Tech Research Center" Project for Private Universities: matching fund subsidy from the Minister of Education, Culture, Sports, Science and Technology, 2004–2008.

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