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Characterization of a group I intron in the nuclear rDNA differentiating *Phialophora gregata* f. sp. *adzukicola* from *P. gregata* f. sp. *sojae*

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An insertion sequence was detected near the 3′ end of the nuclear small subunit rDNA in isolates of *Phialophora gregata* f. sp. *adzukicola*, the causal agent of the brown stem rot disease of adzuki bean. This insertion sequence was absent in isolates of *P. gregata* f. sp. *sojae* which causes brown stem rot of soybean. The insertion sequence is 304 bp long and contains all the characteristics of group I introns. These characteristics include the four conserved sequence elements (P, Q, R, and S), a U at the 5′ splice site of the exon, a G at the 3′ splice site of the intron, a putative internal guiding sequence; the sequence also fits a secondary structure model for group I introns. Similar to most group I introns found in nuclear small subunit rDNA, the intron was located in a highly conserved region and is devoid of long open reading frames. This intron provides a convenient marker for use in conventional PCR to separate *P. gregata* f. sp. *adzukicola* from *P. gregata* f. sp. *sojae*.

Key Words——brown stem rot; group I intron; molecular identification; ribosomal DNA.

The soilborne deuteromycete *Phialophora gregata* (Allington et Chamberlain) Gams causes brown stem rot of adzuki bean, mung bean and soybean (Kobayashi et al., 1983, 1991; Gray and Pataky, 1994). Isolates of *P. gregata* from both adzuki bean and soybean infect mung bean (Kobayashi et al., 1991; Gray and Pataky, 1994). However, isolates obtained from adzuki bean do not infect soybean and vice versa (Kobayashi et al., 1991). Consequently, Kobayashi et al. (1991) proposed two formae speciales, *P. gregata* f. sp. *adzukicola* Kobayashi, Yamamoto, Negishi et Ogoshi and *P. gregata* f. sp. *sojae* Kobayashi, Yamamoto, Negishi et Ogoshi for the isolates obtained from adzuki bean and soybean, respectively. In addition to the difference in pathogenicity, the two formae speciales can be differentiated based on isozyme banding patterns (Yamamoto et al., 1990), restriction banding patterns of mitochondrial DNA (Gray and Hepburn, 1992; Yamamoto et al., 1993), GC content and DNA sequence homology (Yamamoto et al., 1992), genome sizes and the amount of repetitive sequences (Yamamoto et al., 1995), different sensitivity to the antimicrobial chemical nystatin (Yamamoto, 1995), and sequence differences in the internal transcribed spacer (ITS) of the nuclear small subunit rDNA (Chen et al., 1996a). During a course of studying genetic variation within *P. gregata*, we discovered that *P. gregata* f. sp. *adzukicola* contained an insertion sequence near the 3′ end of the nuclear small subunit rDNA. This insertion sequence is absent in *P. gregata* f. sp. *sojae*. Here we characterize the insertion sequence found in *P. gregata* f. sp. *adzukicola*.

Materials and Methods

Fungal strains and DNA isolation Three strains each of *P. gregata* f. sp. *adzukicola* and *P. gregata* f. sp. *sojae* were used in this study. The strain numbers are LM1, K5, BSR101 from soybean from USA and 5–22, A8 and ATCC 46906 from adzuki bean from Japan. Mycelia were harvested by filtration from a 2-wk old culture in green bean broth medium (20 g of frozen green bean blended in a Warner blender and filtered through four layers of cheese cloth, in 1 L distilled water). The mycelia were either frozen at −80°C or used immediately for DNA isolation. Mycelia were ground to a fine powder in liquid nitrogen and DNA was isolated from the mycelium powder using the lysis buffer, followed by phenol-chloroform extraction and ethanol precipitation, as previously described (Chen et al., 1996a). DNA concentration was estimated by agarose gel electrophoresis by comparing with known mass of standard DNA.

Polymerase chain reaction and DNA sequencing Polymerase chain reaction (PCR) was carried out using primers NS7 (5′GAG GCA ATA ACA GGT CTG TGA

TGC3′) and NS8 (5′TCC GCA GGT TCA CCT ACG GA3′) located in the nuclear small subunit rDNA (White et al., 1990). The combination of these two primers amplified a region of DNA near the 3′ end of the small subunit rDNA. PCR conditions are 50 μM each of dATP, dCTP, dGTP, and dTTP, 0.8 μM each of the primers, 2.5 units of *Taq* DNA polymerase (GIBCO-BRL), and about 1 ng DNA template in 100 μl reaction. Temperature cycling parameters were denaturation at 94°C for 2 min for the first cycle and 45 s for subsequent cycles, primer annealing at 50°C for 45 s and primer extension at 72°C for 1 min for 30 cycles plus a final extension for 3 min at 72°C, which were controlled by the PTC-100 programmable thermal controller with heated lid (MJ Research). The efficacy of PCR was checked by using agarose gel electrophoresis and compared with the 1 kb DNA ladder (GIBCO-BRL).

For DNA sequencing, the PCR products of isolates ATCC 46906, K5 and LM1 were first purified by agarose gel electrophoresis in TAE buffer to remove unincorporated primers and nucleotides, then the DNA in the agarose slice was further purified using the Elu-Quick DNA purification kit (Schleicher and Schuell). The concentration of purified DNA was estimated using agarose gel electrophoresis before used in DNA sequencing. The sequencing reactions were carried out using the Thermo Sequenase dye terminator kit (Amersham Life Science). About 100 ng of purified DNA and 5 μM of one of the PCR primers were added to the sequencing reagent premix in a total of 20 μl without oil overlay. The temperature conditions for the cycle sequencing reaction were 96°C for 1 min then 30 cycles of 30 s at 96°C, 15 s at 45°C and 4 min at 60°C in the PTC-100 programmable thermal controller. After sequencing reactions, the unincorporated terminator dyes were removed by using ethanol precipitation in the presence of ammonium acetate according to the manufacturer's instructions. After dissolving in loading dye and denaturation, the samples were then loaded onto the sequencing gel for ABI 377 automated sequencer at the Biotechnology Center of the University of Illinois at Urbana-Champaign. The chromatograms from the automated sequencer were then input into the computer program Sequencer 3.0 (Gene Codes) for sequence editing and assembly. The PCR products from the three isolates were completely sequenced in both strands.

Intron Analyses The intron location (the intron-exon splice site) in *P. gregata* f. sp. *adzukicola* was predicted by sequence comparisons with isolates of *P. gregata* f. sp. *sojae* in which the intron is lacking, and by comparing with the same region of other published sequences in GenBank. Computer database searches for similar sequences, with the exon and the intron sequences as queries, were performed with the Basic Local Alignment Search Tool (BLAST) algorithm (Altschul et al., 1990) at the National Center for Biotechnology Information (Bethesda, Maryland). A secondary structure model of the putative group I intron sequence found in the isolate ATCC 46906 of *P. gregata* f. sp. *adzukicola* was predicted according to the structure conventions for group I introns (Burke et al., 1987). Secondary structures within loops were resolved with the energy minimization protocol of the computer program MulFold version 2.0 (Jaeger et al., 1989, 1990; Zuker, 1989). The secondary structure was drawn with the program loopDloop (Gilbert, 1992) and finally modified in MacDrawPro (Claris Corporation).

Results

Identification of the intron PCR amplification with primers NS7 and NS8 showed length variation among the 6 isolates used in this study. The PCR product is about 300 bps long in isolates of *P. gregata* f. sp. *sojae*, but about 600 bps long in isolates of *P. gregata* f. sp. *adzukicola* (Fig. 1). Sequence analysis showed that the sequence in the *P. gregata* f. sp. *adzukicola* is identical to that in *P. gregata* f. sp. *sojae* except a 304 bp insertion in the sequence from the isolate of *P. gregata* f sp. *adzukicola* (Fig. 2). The nucleotide sequences of isolates LM1, K5 and ATCC 46906 were deposited in GenBank and assigned accession numbers AF056485, AF056486 and AF056487, respectively. The 376-bp exon sequence has greater than 90% similarity with more than 100 sequences of fungal nuclear small subunit rDNA based on a BLAST search. A BLAST search with the 304-bp intron sequence as query identified segments ranging from 40 to 120 nucleotides within the intron sequence showing more than 67% similarity with 44 intron sequences located in mostly nuclear but also some mitochondrial ribosomal DNA sequences. Most of the regions showing

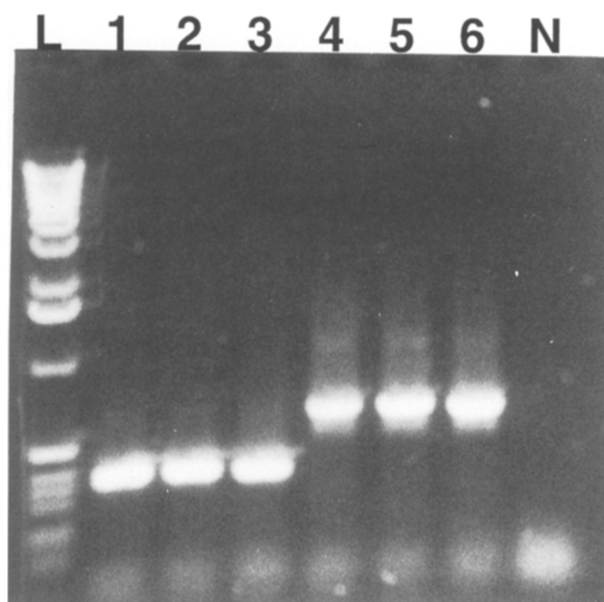

Fig. 1. Agarose gel showing the products of PCR amplification with primers NS7 and NS8.
Lane L: BRL 1 KB DNA ladder; Lanes 1 to 3: isolates of LM1, K5, and B2 of *Phialophora gregata* f. sp. *sojae*; Lanes 4 to 6: isolates of 5–22, A8 and ATCC 46906 of *P. gregata* f. sp. *adzukicola*; Lane N: negative control without template DNA.

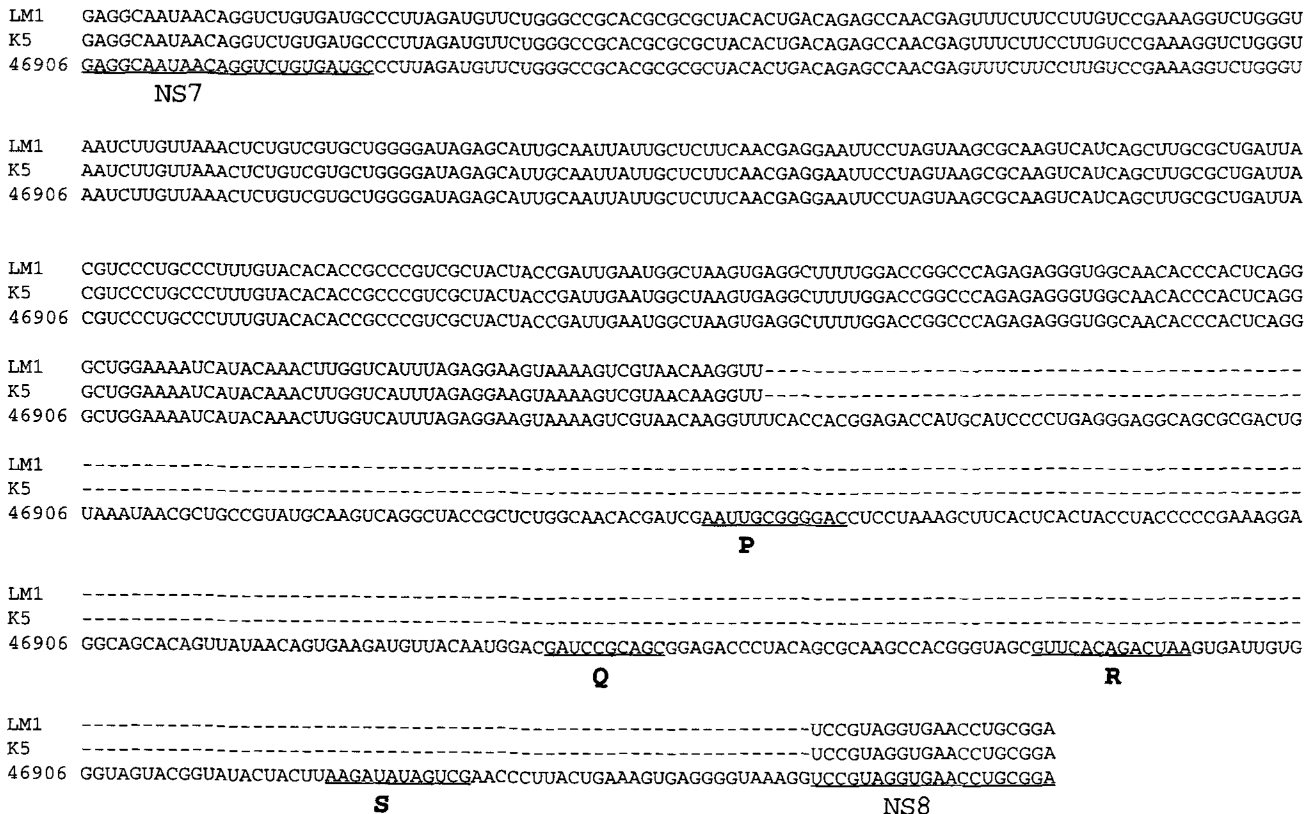

Fig. 2. Alignment of RNA sequences deduced from DNA of the amplified region between PCR primers NS7 and NS8 from isolates of LM1 and K5 of *Phialophora gregata* f. sp. *sojae* and isolate ATCC 46906 of *P. gregata* f. sp. *adzukicola*. The conserved sequence elements P, Q, R, and S in the intron sequence are indicated. - indicates an alignment gap. The sequences of the PCR primers NS7 and NS8 are indicated at the beginning and the end of the sequences.

similarity contained one or more of the conserved sequence elements (search results not shown).

Characterization of the intron sequence Analysis of the intron sequence and comparison with other published group I intron sequences showed that the insertion sequence contained sequence features that are known to be highly conserved among group I introns (Fig. 3). These conserved features are a) the insertion sequence has the four conserved sequence elements termed P, Q, R, S which allow the formation of the characteristic group I intron core structure; b) the formation of pairing segments P4, P6, and P7 involves the conserved sequence elements; c) the last exon base immediately upstream of the 5′-intron splice site is a U, and the last base of the insertion sequences at the 3′ splice site is a G; d) there is a putative internal guiding sequence (IGS) near the 5′ splice site that can potentially base pair with the 5′ and 3′ of the exons forming P1 and P10; thus, the IGS brings the nucleotides boarding the spice site to close proximity. Additionally, the U at the 5′ splice site is base-paired with a G of the IGS. Other features of this intron include: pairing segment 6 (P6) of the potential secondary structure begins with a G–U pair followed by a C–G pair; the J6/7-1 and 2 (the first and the second bases between P6 and P7) are U and C bases; a bulged A in P7 which is immediately followed by a G–C pair. Also the intron sequence is devoid of any lengthy open reading frames (the longest ORF is 25 amino acids long), similar to most of the group I introns in nuclear small subunit rDNA.

Discussion

The presence of the group I intron in *P. gregata* f. sp. *adzukicola* provide an easily identifiable marker for differentiating this forma specialis from *P. gregata* f. sp. *sojae*. The two formae speciales are morphologically identical, but a number of physiological and genetic differences between these two formae speciales have been reported (Yamamoto et al., 1990, 1992, 1995; Gray and Hepburn, 1992; Chen et al., 1996a). Considering the feature of concerted evolution of nuclear rDNA within populations (Arnheim et al., 1980; Dover and Coen, 1981), the difference in the presence of this intron indicates separate evolution of the two populations, providing another line of evidence to support the separation of the two formae speciales. While studying the population genetics of *P. gregata*, we found two microsatellite loci that can unequivocally differentiate the two formae speciales and it seems that there is more genetic variation in *P. gregata* f. sp. *adzukicola* than in *P. gregata* f. sp. *sojae* in one of the microsatellite loci (unpublished information). *P. gregata* f. sp. *adzukicola* was

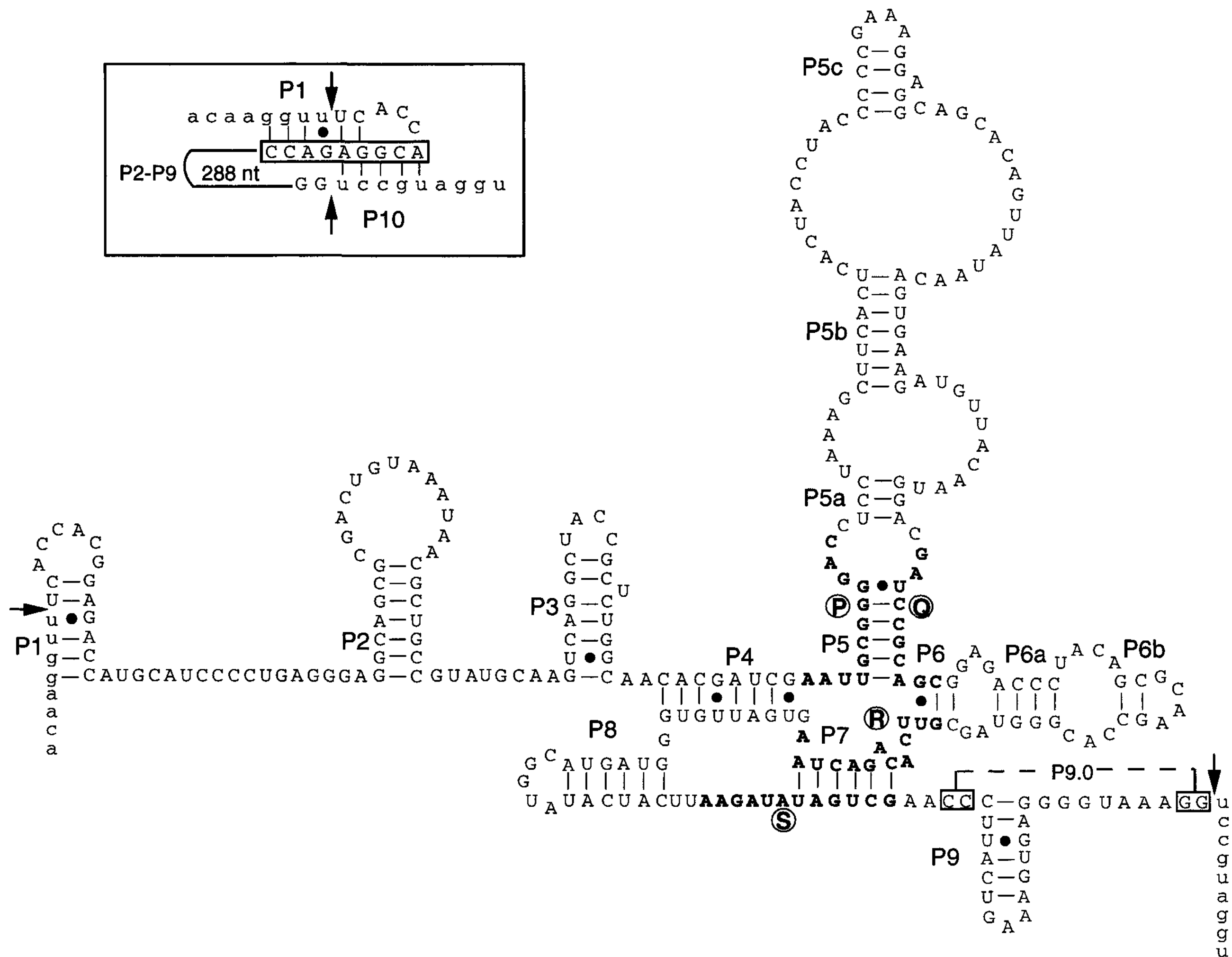

Fig. 3. Potential secondary structure model of the intron sequence found in the nuclear small subunit rDNA of the isolate ATCC 46906 of *Phialophora gregata* f. sp. *adzukicola*.
Intron sequences are in uppercase letters, and exon sequences are in lowercase letters. Arrows indicate putative splice sites. Bold type letters indicate conserved sequence elements labeled as circled P, Q, R, and S that are found the group I introns. Inset: the exon pairing with putative internal guiding sequence (boxed) forming pairing elements P1 and P10 potentially bringing the ends of exons together for rejoining after intron splicing.

shown to be more variable in banding patterns of esterase and peroxidase than *P. gregata* f. sp. *sojae* in Japan (Yamamoto et al., 1990). The information on genetic variation suggests that *P. gregata* f. sp. *sojae* could be a more recently derived population of *P. gregata* than *P. gregata* f. sp. *adzukicola*.

Group I introns are widespread in the nuclear, mitochondrial and chloroplast genomes, in bacterial-phages that infect gram-positive and gram-negative bacteria and in eukaryotic viruses (Cech, 1990; Michel and Westhof, 1990; Yamada et al., 1994). In the nuclear genome, group I introns are reported to occur only in the rDNA. In the nuclear small subunit rDNA of fungi, group I intron was first reported in *Pneumocystis carinii* initially believed to be a protozoan (Sogin and Edman, 1989). Group I introns have since been reported from a number of fungi (see DePriest, 1993; Nishida et al., 1993; Gargas et al., 1995; Chen et al., 1996b). Here we report a group I intron in a deuteromycete whose phylogenetic affiliation with sexual fungi is still unknown.

Group I introns are believed to be mobile genetic elements that encode sequence-specific double stranded endoDNases to facilitate their propagation and spreading (Dujon, 1989). The intron found in *P. gregata* f. sp. *adzukicola* is probably no longer mobile because like most introns found in the nuclear small subunit rDNA it lacks any long open reading frames. The uniqueness of this group I intron in all adzuki bean isolates remains to be verified, although it was absent from all soybean isolates obtained from various states of the US and from Brazil used in a previous study (Chen et al., 1996a). Nevertheless, the intron provides a convenient molecular marker for use in conventional PCR to identify and separate *P. gregata* f. sp. *adzukicola* from *P. gregata* f. sp. *sojae*.

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