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Expression of Reverse Transcriptase Genes in *Fulvia fulva*

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Antibodies raised against intercellular fluid antigens isolated from diseased tomato leaves have revealed that the fungal pathogen *Fulvia fulva* expresses genes for a fungal reverse transcriptase (RNA-dependent DNA polymerase). This enzyme is required for the replication of retroviruses and retroviral-like transposable elements and could provide a mechanism for increasing the mutation rate of fungal pathogens, perhaps explaining their ability to evolve new races rapidly. We report here the DNA sequence of a 225-bp clone from a λgt11 genomic library of *F. fulva*. This clone, designated P5, exhibits a high degree of sequence homology with the reverse transcriptase (pol) gene of the *Drosophila melanogaster* copia-like retrotransponson *17.6*. Southern blot analysis of genomic DNA of *F. fulva* showed that P5-related sequences are moderately reiterated with 30–100 copies, some of which exhibit restriction fragment length polymorphism in different races of the pathogen. Western blot analysis of extracts from *F. fulva* with antibodies raised to purified reverse transcriptase (from human immunodeficiency virus-1) revealed immunoreactive proteins. Reverse transcriptase previously has been detected in a variety of organisms including yeast, insects, protozoa, and mammals, but to our knowledge, this is the first report of its occurrence in filamentous fungi.

Additional keywords: *Lycopersicon esculentum*, tomato leaf mold.

The ability of fungal plant pathogens to rapidly evolve new virulent races capable of infecting previously resistant plant cultivars has frequently been noted. Mutation rates to virulence as high as 10^{-3} per generation have been observed in, for example, *Puccinia coronata* (reviewed in Dinoor *et al.* 1988). These high mutational rates suggest that transposable elements might be involved. However, no evidence yet exists for their presence in any fungal plant pathogen or indeed in any filamentous fungus. The recent observation by Kearney *et al.* (1988) of inactivation of an avirulence gene by insertion of a transposable element in the bacterial pathogen *Xanthomonas campestris* pv. *vesicatoria* is the first evidence for the involvement of transposable elements in a plant-pathogen interaction.

Fulvia fulva, the causal agent of tomato leaf mold, is well suited to studies of race specificity because it exists in a number of races characterized by their abilities to infect differentially tomato isolines containing various Cf resistance (*R*) genes (Boukema 1981). Compatible interactions result from the inoculation of races containing virulence genes onto cultivars containing only the corresponding Cf *R* genes. The pathogen is restricted to the intercellular space, so apoplastic fluids have been studied on the assumption that these contain fungal products contributing to pathogenicity and cultivar specificity (De Wit 1977; De Wit and Spikman 1982; De Wit *et al.* 1984, 1986).

The aim of the current study was to identify fungal gene products exported into the apoplast. We have used antibodies, raised against substances isolated from intercellular spaces of infected leaves, to screen a λgt11 (Young and Davis 1983) library of genomic DNA of *F. fulva*. We describe here the isolation of P5, a cloned DNA sequence of *F. fulva*, which shows strong homology to viral and retrotransposon reverse transcriptase sequences. We also present immunological evidence for the active expression of these genes.

Nucleotide and/or amino acid sequence data is to be submitted to GenBank as accession number J03675.

MATERIALS AND METHODS

Maintenance of fungal cultures. *F. fulva* (Cooke, Ciferri) (syn. *Cladosporium fulvum* (Cooke)) and tomato cultivars were grown as described previously (Harling *et al.* 1988; De Wit and Flach 1979).

λgt11 cloning and sequence analysis of P5. DNA was isolated from mycelium as described (Oliver *et al.* 1987; Raeder and Broda 1985). DNA from race 4 of *F. fulva* was digested under conditions that gave *Eco*RI "star" fragments with a maximum size of 7 kb; that is, 10 μg of DNA was incubated at 37° C for 22 hr in 100 μl of 25 mM Tris-Cl buffer, pH 8.5, containing 20% glycerol, 8% DMSO, 2 mM $MgCl_2$, and 500 units of *Eco*RI (Woodbury *et al.* 1980).

High titer λgt11 was prepared by plate lysates, (Young and Davis 1983) and purified on two cesium chloride gradients; phage DNA was extracted by formamide dialysis. A library of 2×10^6 recombinant phage was constructed by ligating 1 μg of *Eco*RI-cut, phosphatased λgt11 with 1 μg of *Eco*RI star-digested genomic DNA of *F. fulva* and packaging with Giga-pack-gold (Stratagene). The library was screened by using antibodies according to Young and Davis (1983), except that goat-anti-rabbit horseradish-peroxidase and 4-chloro-1-naphthol (both from Sigma, St. Louis, MO) were used for visualization. Positive plaques were purified, and the insert of one, P5, was subcloned into pUC18 (Norrander *et al.* 1983) and sequenced (Chen and Seeburg 1985). The DNA sequence, and the predicted amino acid sequence of the single open-reading frame, were compared with sequences in the EMBL, GenBank, and PIR databases.

Southern analysis. DNA from each race was analyzed by Southern blotting as described (Oliver *et al.* 1987). Two μg of DNA was digested with restriction enzymes according to

manufacturer's instructions (Bethesda Research Laboratories or Pharmacia) and electrophoresed on an 0.8% agarose gel. After transfer to Hybond-C (Amersham; according to manufacturer's instructions), the filter was hybridized to ^{32}P-labeled P5 insert by using the Amersham Multiprime Kit. After hybridization the filter was washed ultimately in 2× saline sodium citrate, 0.1% sodium dodecyl sulfate (SDS) at 65° C for 30 min and autoradiographed by using Fuji X-ray film for 2 days with intensifying screens at −80° C.

Western blot analysis. Protein extracts (Joosten and De Wit 1988), isolated from 7-day shake cultures, were analyzed by SDS-PAGE. Proteins were transferred to nitrocellulose (Sartorius, UK) by using a Sartoblot II semi-dry electroblotter according to manufacturer's instructions. Immunological detection by rabbit anti-reverse transcriptase (RT) polyclonal antisera (1:100 in phosphate-buffered saline) utilized peroxidase-conjugated goat anti-rabbit IgG (1:1,000 in PBS). The anti-RT antibody, kindly provided by M. Tisdale, was raised against bacterially expressed HIV-1 RT after purification by using ion exchange chromatography (Larder and Purifoy 1987).

RESULTS AND DISCUSSION

Our aim was to clone fungal genes encoding proteins present in the intercellular spaces of infected leaves. To create a representative library, genomic DNA was *Eco*RI-star digested before ligation into λgt11. This gave a library of 2×10^6 plaques, and a sample of 10^5 clones was screened with antibody to intercellular substances collected from the race 4/Cf4 compatible interaction. Several positive plaques were identified, and one of these, P5, was selected for further study. Plaques of the purified phage failed to react with antibodies raised to intercellular fluid from the race 5/Cf4 incompatible interaction (data not shown). This represents a combined control for the reaction of preimmune serum and antibody derived from plant antigens and indicated that P5 corresponds to a compatible-specific epitope.

The 225-bp *Eco*RI insert of P5 (Fig. 1a) exhibited highly significant homology with part of the reverse transcriptase sequence of the copialike retrotransposon *17.6* of *Drosophila melanogaster* (Saigo *et al.* 1984). The amino acid and nucleotide sequence homology between P5 and the *17.6* was 48% and 55%, respectively. If allowance is made for the substitution of similar amino acids, 80% homology is observed (Fig. 1b). Furthermore, the P5 sequence includes the eight amino acids most conserved in reverse transcriptase proteins (Toh *et al.* 1983).

P5 therefore appears to be part of a reverse transcriptase gene in the genome of *F. fulva*. Southern blot analysis of the hybridization of the purified P5 insert to *Bgl*II and *Bam*HI digests of genomic DNA prepared from seven races of *F. fulva* (Fig. 2) revealed multiple bands consistent with P5-related sequences being present in 30–100 copies, at a variety of sites within the genome. Different band intensities may be due to sequence variation between some P5-related sequences. These findings are reminiscent of the genomic distribution of retroviral and retrotransposon sequences (Mellor *et al.* 1986). A comparison of the P5 hybridization pattern to DNA from seven races of *F. fulva* cut with *Bam*HI shows that the majority of bands are invariant (Fig. 2, lanes 7–12), with the exception of a band in the 7- to 9-kb range. The absence of extensive restriction fragment length polymorphism (RFLP) could be explained if the probe were a small, relatively conserved, internal fragment of a large, repeated element.

As a rapid means to assess whether the fungus might be producing active RT, homogenates of race 4 of *F. fulva* were electrophoresed on SDS-polyacrylamide gels, blotted onto nitrocellulose, and probed with a rabbit polyclonal antibody raised to the HIV-1 RT (Fig. 3). This antibody, previously shown to react positively with λgt11 plaques containing P5, revealed three bands of approximately 120, 100, and 60 kDa. No signals were observed when blots were probed with rabbit preimmune serum. The native RT of retroviruses and retrotransposons is believed to be cleaved from the primary pol gene product via a series of intermediates (Garfinkel *et al.* 1985). The mature RT of HIV-1 is approximately 64 kDa (Lightfoote *et al.* 1985). Thus, the bands are consistent with molecular weights of primary and processed retroviral polyproteins. These results also indicate the potential use of antibodies to heterologous RT to identify retroelements in other species.

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P5 CLONE     GAATTCTTGGTCATGCCAATGGGATTGACCAACGCACCCG
             :::: ::: :::::::: : ::::: :  ::::: :: :
COPIA 17.6   GAATATTTGCGCATGCCATTCGGATTAAAAAACGCGCCAG

             CATCCTGCCAGGACCTTGTCAACGAAACACTTAGAGACCT
             :  ::: ::       : :: :: :   : :::   ::
             CCACCTTTCAACGGTGCATGAATGATATTTTAAGACCACT

             ACTCGACGTGTGCGTCGTTGTCTACATGGACGACATACTG
               :  ::     :    :::: ::  :::::::::: :
             CTTAAACAAACACTGTCTTGTGTATTTGGACGACATAATT

             GTCTACACAAAAGGATCCCTCCAGGAACATACCAAGCAAG
             :: : : : : :   :::::  : :::::     :
             GTATTCTCGACA---TCCCTTGATGAACACCTGCAATCGC

             TTCAAGATGTGTTCGAACGACTCACGAAGTCCGGATTCAA
             :   :   :: ::::::  : :  : ::  ::    : ::
             TCGGACTAGTTTTCGAAAAATTAGCAAAAGCCAACCTTAA

             GACAGCACCCGAGAAATGCGAATTC
                :  ::  :: ::::: :: ::
             ATTACAACTTGACAAATGTGAGTTT
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P5                          EFLVMPMGLTNAPASCQDLVNETL----R
COPIA 17.6                  EYLRMPFGLTNAPAFTQRCMNDIL----R
HIV                         IYKVLPQGWKGSPAIFQHTMRQVLEPF-R
ARV-2                       QYNVLPQGWKGSPAIFQSSMTKILEPF-R
VISNA LENTIVIRUS            YWKVLPQGWKLSPAVYQFTMKQIL----R
HAM. INTRACISTERNAL A       QWKVLPQGMANSPTICQLYVQEALEPIRR
ROUS SARCOMA VIRUS          QWKVLPQGMTCSPTICQLVVGQVLEPL-R
CaMV                        EWNVVPFGLKQAPSIFQRHMDEAF----R
EQU. INFECT. ANEMIA VIRUS   VWKCLPQGFVLSPYIYQKTLQEILQPF-R
MOLONEY MURINE VIRUS        TWTRLPQGFKNSPTLFDEALHRDLLADFR

-D-LLDVCVVV----YMDDILVYTKGSLQEHTKQVQDVFERTLKSGFKTAPEKCEF
-P-LLNKHCLV----YLDDIIVFS-TSLDEHLQSLGLVFEKLAKANLKLQLDKCEF
KA-NKDVIIIQ----YMDDILIASDRTDLEHDRVVLQLKELLNGLGFSTPDEKFQK
KQ-NPDIVIYQ----YMDDLYVGSDLEIGQHRTKIEELRQHLLRWGFTTPDKKCEF
-WIEEHMPIMIQFGIYMDDIYIGSDLGLEEHRGIVNELASYIAQUGFMLPEDKCEF
KQ-FTSLIVIH----YMDDILICHK-ELDVLQKAFPMLVAELKQWGLEIASEKQVI
-L-KHPSLCMLH---YMDDLLLAA-SSHDGLEAAGEEVISTLERAGFTISPDKVQR
-V-FRKFC-CV----YVDDILVFSNNE-EDHLLHVAMILQRCNQHGIILSKKKCEF
-ERYPEVQLYQ----YMDDLFVGSNGSKKQHKELIFELRAILQK-GFETPDDKLQE
-I-QHPDLILLQ---YVDDLLAAS-TSELDCQQGTRALLQTLGNLGYRASAKKAQI
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Fig. 1. A, Alignment of the DNA sequence of P5 with that of copialike 17.6 reverse transcriptase (RT). **B**, Optimal alignment of inferred P5 amino acid sequence with several known reverse transcriptases. Amino acids in bold type are invariant among RTs sequenced to date. The percentage of similarity was calculated by using the following amino acid groupings: A,S,T,P,G; N,D,B,E,Q,Z; H,R,K; M,L,I; and I,Y,W.

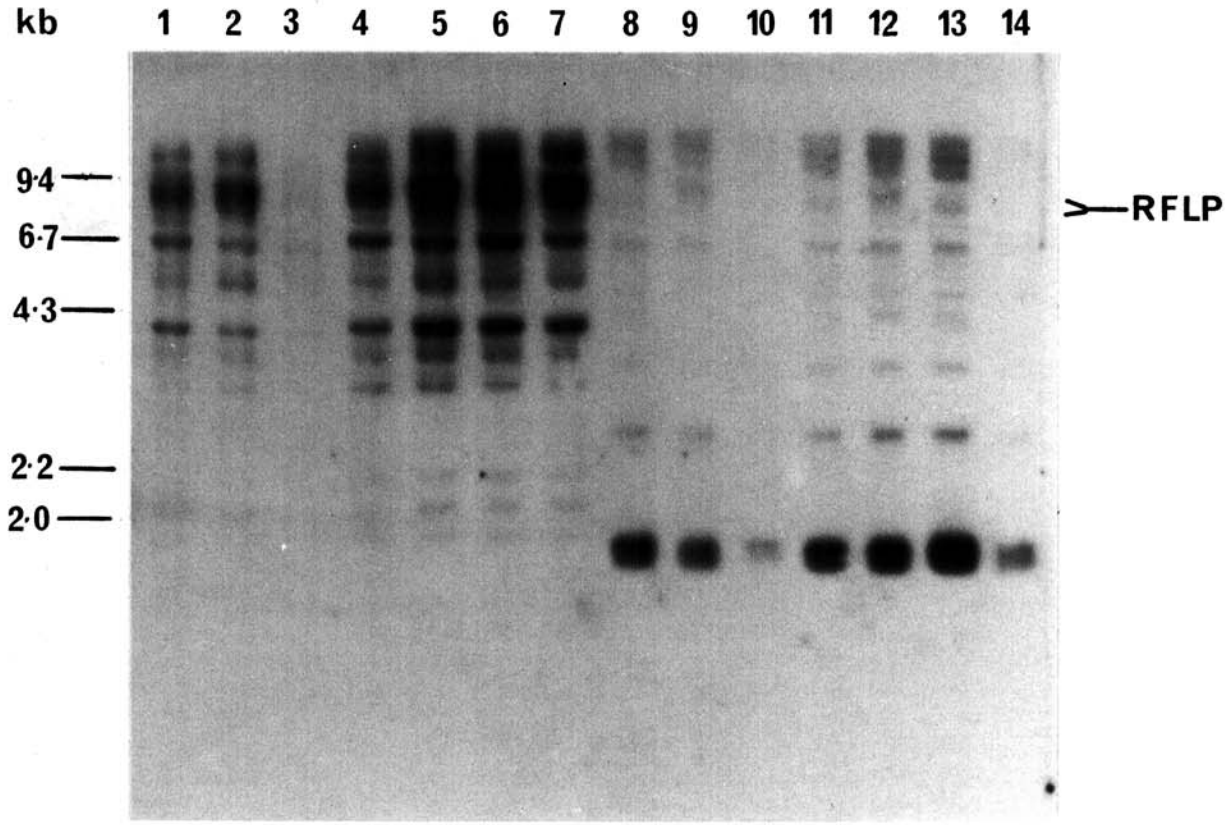

Fig. 2. Southern blot analysis of genomic DNA of *F. fulva* probed with the purified P5 insert. DNA from seven races (races 0, 2, 4, 2.4, 5, 2.4.5, and 2.4.5.9, respectively) was digested with *Bgl*II (lanes 1–7) and *Bam*HI (lanes 8–14). Arrows indicate a restriction fragment length polymorphism in the 7- to 9-kb region.

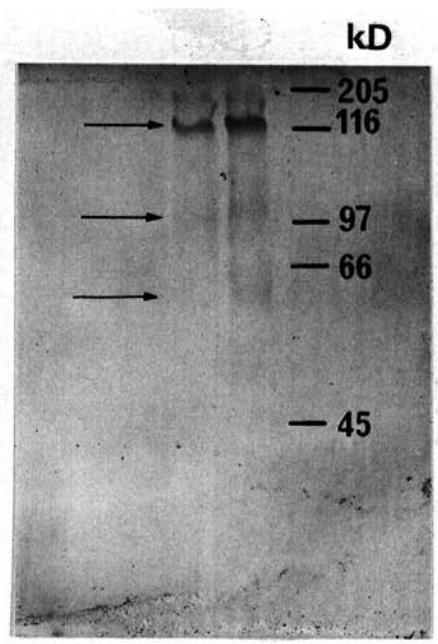

Fig. 3. Western blot analysis of mycelial extracts from separate cultures of *F. fulva* probed with antibody to HIV-1 RT. Arrows indicate positions of positive signals.

Retroviruses and retrotransposons are often associated with 40–120 nm virus-like particles (VLPs). Particles in the range of 50–60 nm, with a morphology similar to yeast Ty VLPs (Garfinkel *et al.* 1985) were observed in sucrose gradient fractions of fungal homogenates (data not shown). Further experiments to assay for VLP-associated RT, both by Western blot and by enzyme activity, are in progress. It should be noted, however, that the presence of RT epitope in the intercellular fluid is not evidence for the secretion of RT-containing particles by the fungus; hyphal tips may burst during preparation of intercellular fluid (De Wit, personal communication).

The presence of reverse transcriptase genes and of the expressed protein suggests that retroviral or retrotransposon-like elements may be abundant in the genome of *F. fulva*. If the complete element is of a size typical of retrotransposons (i.e., 5–9 kb) the P5-related elements may comprise 0.1–0.5% of the genome of *F. fulva*. Should the elements prove to be transposable, the mutagenic effects of replicative transposition may provide a mechanism for generating variability that may be important in the maintenance of virulence by fungal pathogens in response to the introduction of resistant varieties of crop species and fungicides.

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