PCR Amplification and Sequence Analyses of Reverse Transcriptase-like Genes in *Crinipellis perniciosa* Isolates

Jorge F. Pereira[1], Mariana D.C. Ignacchiti[1], Elza F. Araújo[1], Sérgio H. Brommonschenkel[2], Júlio C.M. Cascardo[3], Gonçalo A. G. Pereira[4] & Marisa V. Queiroz[1]

[1]Departamento de Microbiologia, BIOAGRO; [2]Departamento de Fitopatologia, BIOAGRO, Universidade Federal de Viçosa, CEP 36571-000, Viçosa MG, Brazil; [3]Departamento de Ciências Biológicas, Universidade Estadual de Santa Cruz, CEP 45650-000, Ilhéus BA, Brazil; [4]Departamento de Genética e Evolução, Universidade Estadual de Campinas, CEP 13083-970, Campinas SP, Brazil; e-mail: mvqueiro@ufv.br

Author for correspondence: Marisa V. Queiroz

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ABSTRACT

Reverse transcriptase (RT) sequence analysis is an important technique used to detect the presence of transposable elements in a genome. Putative RT sequences were analyzed in the genome of the pathogenic fungus *C. perniciosa*, the causal agent of witches' broom disease of cocoa. A 394 bp fragment was amplified from genomic DNA of different isolates of *C. perniciosa* belonging to C-, L-, and S-biotypes and collected from various geographical areas. The cleavage of PCR products with restriction enzymes and the sequencing of various RT fragments indicated the presence of several sequences showing transition events (G:C to A:T). Southern blot analysis revealed high copy numbers of RT signals, forming different patterns among C-, S-, and L-biotype isolates. Sequence comparisons of the predicted RT peptide indicate a close relationship with the RT protein from the *gypsy* family of LTR-retrotransposons. The possible role of these retrotransposons in generating genetic variability in the homothallic *C. perniciosa* is discussed.

Additional keywords: genetic variability, transposable elements, witches' broom, *Theobroma cacao*.

RESUMO

Amplificação por PCR e análises de seqüências de genes do tipo transcriptase reversa em isolados de *Crinipellis perniciosa*

A análise de sequências de transcriptase reversa (RT) é uma etapa importante para descobrir a presença de elementos transponíveis e investigar o seu papel na geração de variabilidade genética em *C. perniciosa.* Seqüências putativas de TR foram analisadas no genoma do fitopatógeno *C. perniciosa*, o agente causal da doença vassoura-de-bruxa no cacau. Um fragmento de 394 pb foi amplificado a partir do DNA genômico de diferentes isolados de *C. perniciosa,* pertencentes aos biótipos C, L e S e a distintas áreas geográficas. A clivagem dos produtos de PCR com diferentes enzimas de restrição e sequenciamento de vários fragmentos de TR indicou a presença de diferentes seqüências mostrando eventos de transição G: C para A:T. A análise por hibridização revelou alto número de sinais sugerindo a presença de cópias de TR com diferentes perfis entre os isolados dos biótipos C, S e L. As comparações de seqüências dos peptídeos preditos indicam uma relação próxima com a proteína TR de retrotransposons-LTR da família *gypsy*.

Palavras-chave adicionais: variabilidade genética, transposons, vassoura de bruxa, *Theobroma cacao*.

INTRODUCTION

Reverse transcriptase (RT) is a fundamental enzyme for the transposition of Class I transposable elements. There are two major groups of Class I retrotransposons, one that contains long terminal repeats (LTR retrotransposons – *Gypsy/Ty3-like* and *Copia/Ty1-like*) and the other that lacks LTRs and possesses a polyadenylate sequence at its 3' termini (non-LTR retrotransposons – *LINE-like* and *SINE-like*). The first retroelement reported for filamentous fungi was the *Tad* element of *Neurospora crassa* Shear & B.O. Dodge isolated via an insertion into glutamate dehydrogenase gene (Kinsey & Helber, 1989). Over the last 15 years, more than 30 retroelements were reported in different species of filamentous fungi, mainly from poorly characterized species and, in most cases, without a described sexual cycle (for a review, see Daboussi and Capy, 2003).

These retroelements present an extraordinary potential to promote modifications through their insertion and excision mechanism, as well as for the recombination

Present Address of the first author: Embrapa Trigo, Cx. Postal 451, CEP 99001-970, Passo Fundo RS, Brazil.

between dispersed elements in the genome (see reviews in Daboussi & Capy, 2003; Kazazian Jr., 2004). These modifications are essential for the generation of genetic variability within a species. In phytopathogenic fungi, the studies of these elements can increase the knowledge of the genetic structure of natural populations (Daboussi, 1997).

Although transposable elements are widespread in the eukaryotic genome, many of them are inactive. One mechanism that is responsible for the irreversible inactivation of duplicated sequences, such as transposable elements, was found in *N. crassa*. This mechanism is known as RIP (repeat-induced point mutation) and efficiently detects duplications of gene-sized DNA segments, linked or unlinked, and generates G:C to A:T mutations in both copies of the duplicated DNA (Selker & Garret, 1988; Cambaberi *et al*., 1989). RIP-like processes have been previously identified in other fungi, such as *Aspergillus fumigatus* Fresen., *Magnaporthe grisea* (T.T. Hebert) M.E. Barr, *Podospora anserina* (Rabenh.) Niessl*, Leptosphaeria maculans* (Desm.) Ces. & De Not*., Microbotryum violaceum* (Pers.) G. Deml & Oberw. and *Ophiostoma* Syd. (Neuvéglise *et al*., 1996; Nakayashiki *et al*., 1999; Hamann *et al*., 2000; Hood *et al.*, 2005; Attard *et al*., 2005; Bouvet *et al*., 2007), and constitute important protection mechanisms against the deleterious effects of transposition (Kinsey *et al.*, 1994; Galagan & Selker, 2004).

Crinipellis perniciosa is a basidiomycete (*Agaricales, Tricholomataceae*), causal agent of witches' broom in cocoa tree (*Theobroma cacao*). According to pathogenicity data, this species is subdivided into three biotypes: (1) C-biotype, infecting *Theobroma* and *Herrania* species (Sterculiaceae) (Evans, 1978; Bastos *et al.*, 1988); (2) S-biotype, infecting a number of species of the family *Solanaceae* (Bastos & Evans, 1985); and (3) L-biotype, a saprophyte that colonizes a variety of substrates (Evans, 1978; Hedger *et al*., 1987). Witches' broom is considered one of the most severe cocoa diseases and has accounted, in the last two decades, for the drop in Brazilian cocoa production, turning the country from being one of the world's main cocoa exporters to a net importer. Because of the great impact of this disease on the main cocoa producing regions of Brazil, a consortium comprising a number of Brazilian institutions was created to sequence the genome of *C. perniciosa* (www.lge.ibi.unicamp.br/vassoura). Search analysis by sequence comparison in the *Crinipellis* Genome database revealed the presence of sequences showing homology to RT sequences from other organisms.

In this paper, the presence and distribution of putative reverse transcriptase sequences in isolates of *C. perniciosa* from different biotypes and geographical areas were analyzed. PCR and Southern analyses led to the identification of different putative RT sequences in C-biotype isolates and the presence of polymorphic fragments among C, S, and L-biotypes, and between different C-biotype isolates. Possible involvement of the sequences related to transposable elements and the genetic variability of *C. perniciosa* are discussed.

MATERIALS AND METHODS

Fungal strains

The C-, S- and L-biotype isolates of *Crinipellis perniciosa* examined in this study are listed in Table 1. These isolates were grown in PDA (Potate Dextrose Agar) and incubated at 27°C.

Primer construction

Based on a sequence showing similarity to a reverse transcriptase (RT) found in the *C. perniciosa* genome database (GenBank accession nº AY676620), a pair of primers was used to amplify a 394 bp fragment. The primers for RT amplification were RT1 (5'-TGCCAAAGAAACAGGGACTT-3') and RT2 (5'-GAATTTGCGAGACCGAAAAA-3').

DNA extraction, PCR amplification, and restriction analysis

Total DNA of the isolates was extracted as described

TABLE 1 - Isolates of *Crinpellis perniciosa* analyzed in this work

Isolate number	Collection Identification	Biotype	Locality	Host	Responsible Institution*
1	Biotipo L	L	Pichilinge - Equador	*Arrabidaea verrucosa*	UW
2	SABA	C	Santo Amaro BA	*Theobroma cacao*	UFLA
3	CP02	C	Itabuna BA	*Theobroma cacao*	UESC
4	FA 42	C	Barro Preto BA	*Theobroma cacao*	AC
5	FA 277	C	Barro Preto BA	*Theobroma cacao*	AC
6	FA 562	C	Uruçuca BA	*Theobroma cacao*	AC
7	FA 563	C	Barro Preto BA	*Theobroma cacao*	AC
8	DOA-106	S	Juiz de Fora MG	*Solanum lycocarpum*	CEPLAC
9	RWB-551	S	Juiz de Fora MG	*Solanum lycocarpum*	UFV

*UW (University of Wales), in Aberystwyth, U.K.; UFLA (Universidade Federal de Lavras) in Lavras, Minas Gerais, Brazil; UESC (Universidade Estadual de Santa Cruz) in Ilhéus, Bahia, Brazil; AC (Almirante Cacau) in Itajuípe, Bahia, Brazil; CEPLAC (Comissão Executiva do Plano da Lavoura do Cacau) in Ilhéus, Bahia, Brazil; UFV (Universidade Federal de Viçosa) in Viçosa, Minas Gerais, Brazil.

by Specht *et al*. (1982). PCR-amplification was performed with the following program: 40 cycles of 1 min at 94°C, 1 min at 55°C, 1 min at 72°C, and a final extension of 10 min at 72°C. The reactions were prepared in a 25 μL volume, containing 1x thermophilic DNA poly Buffer (Promega), 2.5 mM $MgCl_2$ (Promega), 100 μM each of dNTP, 0.5 μM of each primer, 20 ng of DNA and one unit of *Taq* DNA polymerase (Promega). Negative controls (no DNA template) were used for each set of experiments to test the occurrence of nonspecific amplification. DNA products were analyzed by electrophoresis in a 1.5% (w/v) agarose gel or precipitated for restriction analysis with the restriction enzymes *Ava*I and *Eco*RI. The DNA fragments originated from the cleavage reactions were analyzed in 2% (w/v) agarose gel.

Cloning, sequencing, and sequence analyses

Amplified fragments using total DNA from CP02, SABA, and DOA-106 isolates were cloned into the TOPO vector (Invitrogen Life Technologies) or the pGEM T-Easy vector (Promega), according to the manufacturer's instructions. Sequencing of DNA was carried out by using BigDye® Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems) in a MegaBase 1000 DNA Analysis System (Molecular Dynamics & Life Science). Resulting DNA sequences were used to search the data banks using the BLAST Service Network at NCBI (www.ncbi.nlm.nih.gov/BLAST). Subsequent DNA and deduced protein sequence analyses were carried out using the CLUSTAL W program for multiple alignments (Thompson *et al*., 1994).

Southern blot analysis

Size fractionation of total DNA (3.0 μg) digested with the restriction enzyme *Bam*HI (chosen for not cutting the RT sequence) was performed in a 0.7% agarose gel and transferred onto a Duralon-UV™ membrane (Stratagene), according to standard protocols (Sambrook *et al*., 1989). Hybridization was carried out at 65°C, using as probe a 394 bp DNA fragment with similarity to an RT from isolate CP02. Labelling, hybridization and detection were performed using the "Gene Images™ Random Primer Labelling Module and CDP-Star™ Detection Module" (Amersham), according to the manufacturer's protocols.

RESULTS

Using RT1 and RT2 oligonucleotides, a fragment of expected size (394 bp) was amplified from total DNA of the isolates tested (Figure 1), thus indicating the ubiquity of a putative element carrying the RT sequence in the genome of *C. perniciosa*.

To demonstrate that such oligonucleotides amplify RT sequences belonging to different copies of a putative transposable element, PCR products from the different isolates were cleaved with restriction enzymes and originated polymorphic fragments (Figure 2). These different restriction patterns are related to the presence of several copies of the same element undergoing different modifications. This result was confirmed by the sequencing of the PCR products obtained from isolate CP02 (the same isolate used in witches' broom genome project). Nine cloned fragments were sequenced and compared with the original database sequence (Figure 3). All 10 sequences showed a few differences, sharing 95.4% of total sequence identity. Data also revealed that the differences between the amplified fragments are short, generally single-point mutations. In addition to small insertions and deletions, 51 mutations were characterized as base substitution, with 10 transvertions and 41 transitions (Figure 3).

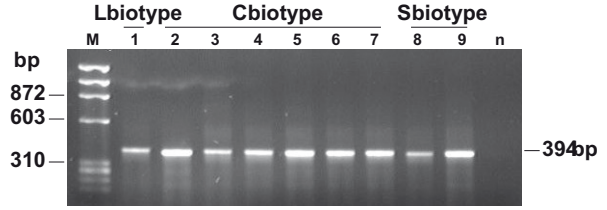

FIG. 1 - PCR products (394 bp) from *C. perniciosa* obtained with primers RT1 and RT2 as described in this work. Numbers 1 to 9 indicate *C. perniciosa* isolates listed in Table 1. Molecular marker in bp is indicated by M (ϕX 174 DNA digested with *Hae*III). N indicates negative control of PCR amplification (mixture reaction without DNA template).

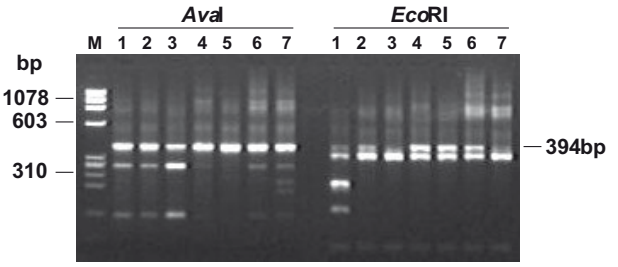

FIG. 2 - Restriction fragments of the PCR-amplified RT sequences from *C. perniciosa* obtained by digestion with *Ava*I and *Eco*RI. Numbers 1 to 7 indicate *C. perniciosa* isolates listed in Table 1. Molecular marker in bp is indicated by M (ϕX 174 DNA digested with *Hae*III).

Southern analyses were conducted to verify the copy number and distribution of RT-like sequences within *C. perniciosa* isolates (Figure 4). The high number of visualized signals suggests that the RT homologous sequences are present in large copy number. Since the signals with higher intensity of hybridization possibly represent elements with more than one copy on the same fragment, it is difficult to determine the exact number of sequences in each isolate. However, distinct profiles were observed for the different biotypes. At least one 4.10 kb fragment was found present in all the isolates analyzed, whereas 5.10, 4.80, and 2.30 kb fragments were found only in the C-biotype isolate. Among C-biotype isolates analyzed, a 1.60 kb polymorphic fragment was present in isolates 3, 5, and 7, but appeared absent in the others,

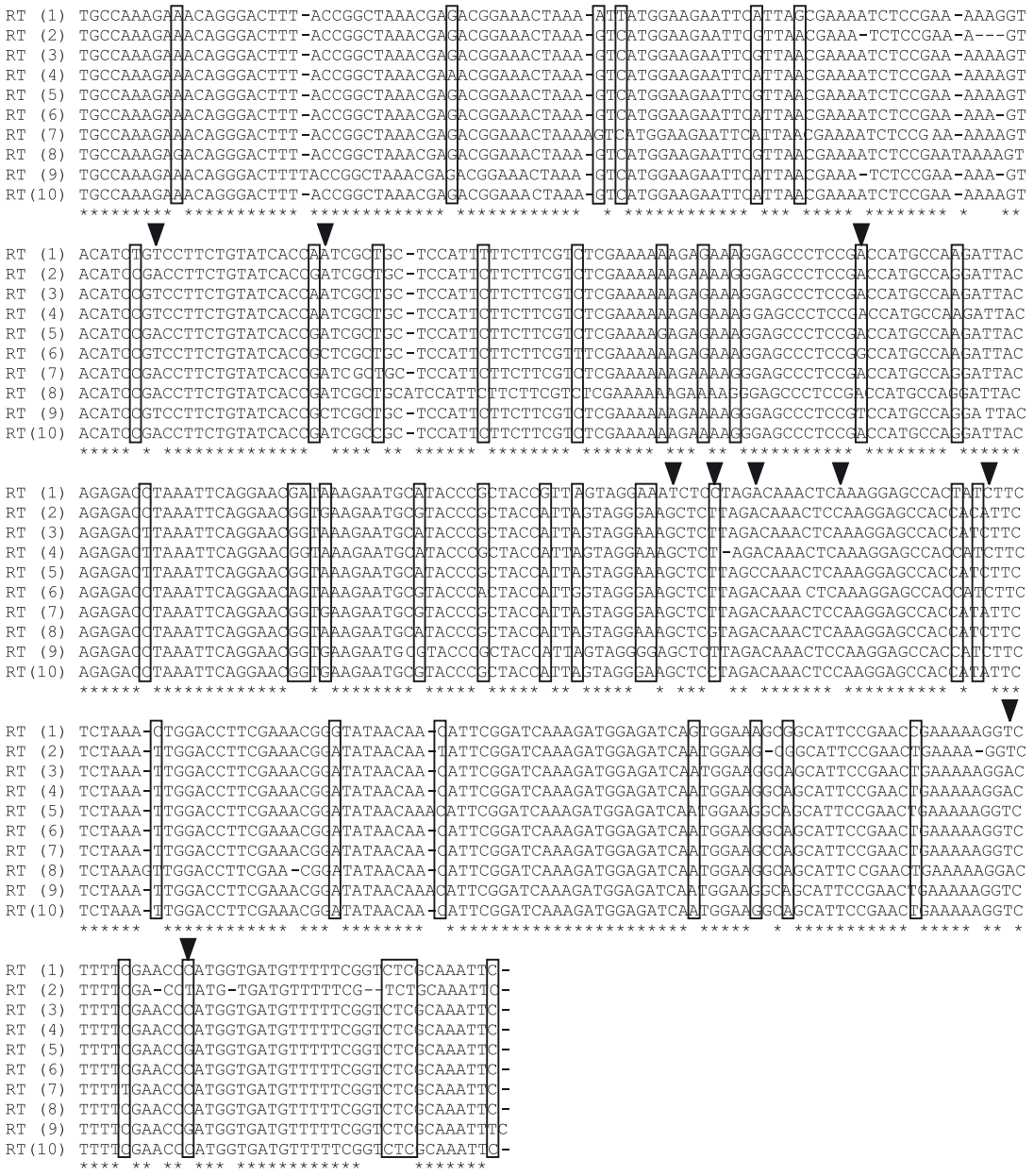

FIG. 3 - Alignment of the RT-like sequences. RT (1) indicates the sequence obtained from the sequencing database project (GenBank n° AY676620); RT (2 to 10) indicates the sequences of fragments obtained in this work (GenBank numbers AY676609, AY676610, AY676611, AY676612, AY676613, AY676614, AY676615, AY676617, AY676618). * indicates presence of the same base in all the sequences; - indicates the presence of gaps. Boxes indicate transitions, and black triangles indicate transvertions.

indicating the presence of at least two hybridization profiles.

The deduced partial amino acid sequence of the putative RT protein was aligned with the homologous proteins from other filamentous fungi (Figure 5). The *C. perniciosa* deduced RT protein showed significant amino acid sequence identity with the RT domains of a number of fungal retrotransposon sequences of the *gypsy* family. The CpC1 RT peptide (RT sequence from isolate 2, GenBank no. AY676620) showed the highest degree of sequence identity (64.6%) with the *PcGR1* RT domain (GenBank AY425728) described in the homobasidiomycete *Phanerochaete chrysosporium* Burds. Other retroelements that share identity with the *C. perniciosa* RT partial protein sequence are *marY1* (GenBank BAA78625) from *Tricholoma matsutake* (S. Ito & S. Imai) Singer (57.6%), *cgRET* (Genbank AAG24792) from *Colletotrichum gloeosporioides* (Penz.) Sacc. (53%), an RT sequence (GenBank BAA92704) from *Tricholoma bakamatsutake* Hongo (52.3%), *MAGGY* (GenBank

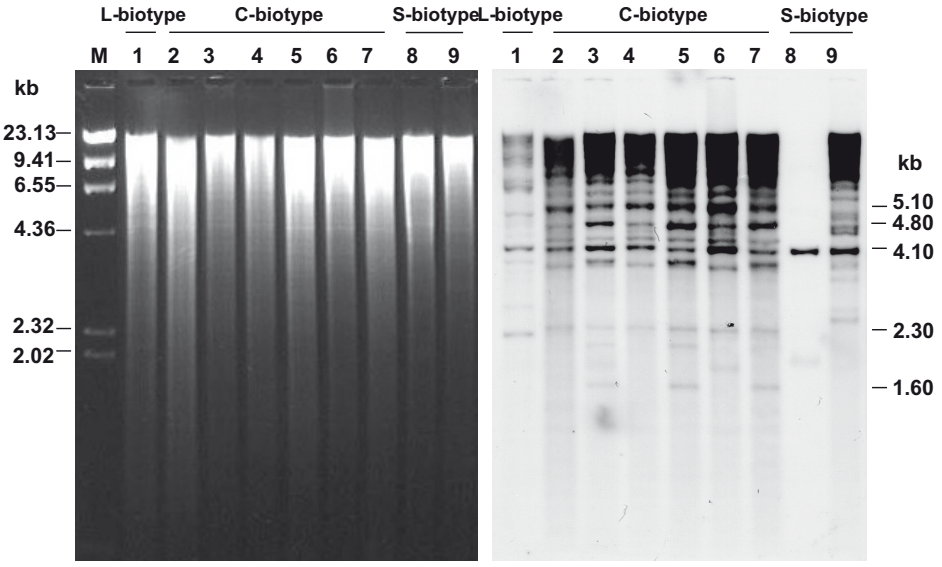

FIG. 4 - Southern blot analysis of total DNA from *C. perniciosa* isolates using the RT amplified sequence as probe. Numbers 1 to 7 indicate *C. perniciosa* isolates listed in Table 1. Total DNA was cut with *Bam*HI and fractionated in 0.7% agarose gel. Molecular size marker λ *Hind*III is indicated in kb on the left. Fragments showed in the right are discussed in the text.

L35053) from *M. grisea* (45.3%), and *Foret* (GenBank X65452) from *Fusarium oxysporum* Schlecht. (36.9%). All of these retroelements belong to the *Ty3/gypsy* group of retrotransposons, indicating that this *C. perniciosa* RT sequence has been amplified possibly from a *gypsy-like* LTR retrotransposon. The amino acid sequences from *C. perniciosa* C-, S-, and L-biotypes share 95% of identity, and the CpC1 and CpC2 RT sequences, belonging to two different C-biotype isolates (isolates 2 and 3 - see Table1), share 94.6% of identity. The CpC1 RT domain shares 93% of identity with the S-biotype RT domain and 85.3% with the L-biotype RT domain.

DISCUSSION

The amplification, distribution, and analyses of putative genes encoding for reverse transcriptase in the witches' broom pathogen, *Crinipellis perniciosa*, are reported in this paper. RT-like sequences were amplified in all tested isolates belonging to different biotypes and geographical areas (Figure 1), which suggest that this sequence is probably an old genome resident of *C. perniciosa*. According to our current hypothesis, the putative element carrying the RT sequence from *C. perniciosa* would have been acquired from a common ancestor of the different biotypes and then inherited vertically by their descendants.

As an old resident of the *C. perniciosa* genome, the putative element carrying this RT sequence may have undergone modifications through time, thus originating different elements. To check for the presence of such different elements, a PCR-amplified product was cleaved and sequenced (Figures 1 and 2), originating different restriction fragments and different sequences, sharing 95.4% identity. The presence of differences in amino acid sequences is a common criterion used to group transposable elements into different families and subfamilies (Capy *et al*., 1998). For instance, the *impala* element of *F. oxysporum* shows approximately 1% differences within each subfamily, but between different subfamilies this difference may exceed 20% (Hua-Van *et al*., 2001). In this context, RT copies amplified from *C. perniciosa* isolates may be related to elements of the same subfamily. The accumulation of alterations may generate transposable elements with defective structures (Daboussi, 1997) that could only be transposed by the reverse transcriptase *in trans* action of an autonomous element. Besides, the transition events G:C to A:T revealed in the different sequences could have resulted from RIP-like (repeated-induced point mutation) processes. Therefore, as revealed by Southern analyses, there are a high number of RT-like copies in *C. perniciosa* genome (Figure 3). Since RIP is a homology-based process that mutates repetitive DNA, the high copy number of retroelements found in *C. perniciosa* would lead to mutations in these repetitive sequences through a RIP-like process, which would control the activity of

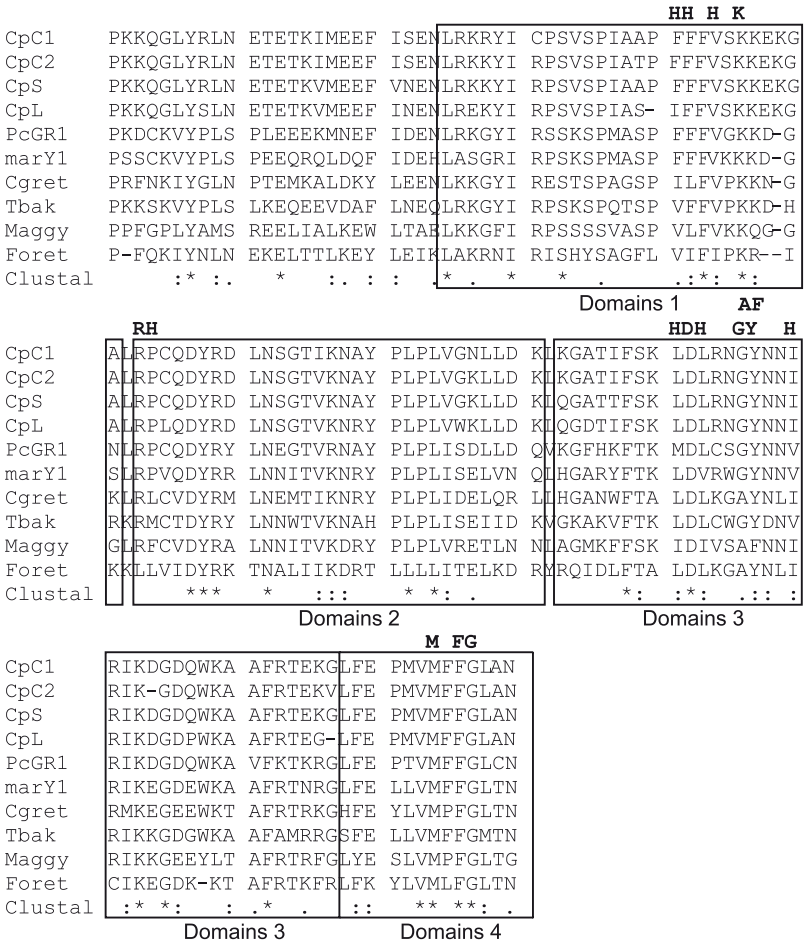

FIG. 5 - Deduced partial amino acid sequence from *C. perniciosa* RT protein aligned with the homologous polypeptides from different filamentous fungi. CpC1 (GenBank n° AY676620) and CpC2 (GenBank n° AY676616) indicate RT from two different *C. perniciosa* C-biotype (isolates 2 and 3 - Table1); CpS (GenBank n° AY676619) indicates RT from *C. perniciosa* S-biotype (isolate 8 - Table 1); CpL (GenBank n° AY842857) indicates RT from *C. perniciosa* L-biotype (isolate 1 - Table 1). Other RT sequences: PcGR1 (GenBank AY425728), marY1 (GenBank BAA78625), CgRET (Genbank AAG24792), Tbak (GenBank BAA92704), MAGGY (GenBank L35053) and Foret (GenBank X65452). Boxes indicate RT domains (1 to 4) identified by Xiong and Eickbush (1990). Some unvaried or chemically similar residues are showed at the top (h, hydrophobic residue; p, small polar residue) as described in Xiong and Eickbush (1990). Hyphens show gaps and asterisks indicate conserved residue.

these retroelements.

The Southern analyses also revealed signal patterns indicating the presence of monomorphic and polymorphic fragments among isolates. The presence of monomorphic fragments corroborates with the hypothesis of a common origin for the *C. perniciosa* RT sequence, and the polymorphic fragments could be related to new insertion events, thus proving the activity of these retroelements.

Moreover, the presence of several copies of retroelements can generate mutations due to the insertion event, but may also serve as substrate for ectopic recombination events between the copies dispersed in the genome, both intra and interchromosomally. Such a recombination event may in turn cause retrotransposon losses (Mieczkowski et al., 2006). This type of mechanism could explain the different profile observed for isolate 8 (Figure 3).

Among the C-biotype isolates analyzed, a 1.60 kb polymorphic fragment was found present in isolates 3, 5, and 7, but absent in the others, thus indicating the presence of at least two different hybridization profiles. In Bahia, the presence of two *C. perniciosa* genotypes was also detected by RAPD (Andebrhan *et al*., 1999) and two different chromosomal patterns were observed by electrophoretic karyotype analysis (Rincones *et al.*, 2003; 2006). According to Andebrhan *et al*. (1999), the geographic distribution of the genetic groups in Bahia and the fact that *C. perniciosa* C-biotype is homothallic suggest the existence of two independent points of introduction of this pathogen into the cocoa-growing region of Bahia. Thus,

the two different RT fingerprint profiles reported here by Southern analysis could also be correlated to the presence of two different genotypes in Bahia, Brazil. Therefore, the RT sequence is another important marker of genetic variability for this fungus.

Furthermore, evidence indicating that transposable elements may play a central role in restructuring evolutionary genomes has been accumulated in a growing number of organisms. In phytopathogenic fungi, the role of these elements has been reported for the genome reorganization of *F. oxysporum* (Davière *et al*., 2000) and in generating DNA fingerprint variation in *M. grisea* (Shull & Hamer, 1996a,b). In *F. oxysporum*, the high level of chromosomal polymorphism was correlated to the concentration of transposable elements resulting from ectopic recombination between dispersed copies (Davière *et al*., 2001). Thus, the widespread presence of RT elements in the genome of *C. perniciosa* may also account for the genetic variability observed in this phytopathogen.

Alignment of the Cp-RT deduced protein with homologous sequences from Genbank revealed that the putative element carrying this sequence belongs to the *gypsy* family of retroelements. The *Ty3/gypsy* family is the most successful group of the retroelements dispersed in the genome of filamentous fungi, since they represent the majority of the 30 retroelements described so far (Daboussi & Capy, 2003). The similarity of the RT protein is higher between the two isolates from C-biotype analyzed (94.6%), and the L-biotype RT protein is more divergent (85.3%) than the S-biotype RT protein (93.0%), when compared with the sequence from C-biotype.

In conclusion, the ubiquity of the RT sequence in *C. perniciosa* biotypes suggests an ancestral origin and vertical inheritance of this sequence by the host progenies. The transposition of the retroelements carrying this sequence generated a high copy number and polymorphic fragments in the different biotypes. Moreover, this activity seems to occur in spite of the presence of several transitions in different RT sequences supporting the existence of a control process. The RT protein analysis indicates that this sequence possibly belongs to a *gypsy-like* retroelement with high similarity with RT from other phytopathogenic fungus. Although the mechanisms for generation of genetic diversity remain to be defined, we showed that sequences related to transposable elements may be important to the genetic variability and evolution in *C. perniciosa*, the most important pathogen of cocoa in Brazil.

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