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RESEARCH ARTICLE

The CD1/CD2 marker for specific detection of *Colletotrichum lindemuthianum* is an iron transporter pseudogene

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ABSTRACT

Colletotrichum lindemuthianum, the causal agent of anthracnose in common bean (*Phaseolus vulgaris*), is one of the most yield-limiting factors worldwide. Anthracnose affects the quality of pods by inducing black, sunken cankers and can also affect petioles, leaf veins and stems where it induces the typical anthracnose sunken lesions. A few years ago, a duplex PCR method that combines amplification of an ITS rDNA segment (CY1/CY2) together with an uncharacterized RAPD-derived amplicon (CD1/CD2) was developed for specific detection of *C. lindemuthianum*. This study shows that the CD1/CD2 marker corresponds to a portion of an iron permease (*Ftr1*) pseudogene in the vicinity of the gene encoding for a polyhydroxyproline-rich protein in *Colletotrichum*. Discrimination with *Colletotrichum orbiculare* is due to a 15 nt deletion in the CY1 annealing region. The potential of using this genomic region for phylogenetic analysis of the *C. orbiculare* species complex and detection of their related species is discussed.
Key words: *Glomerella*, *Phaseolus vulgaris*, anthracnose, PCR-based diagnosis.

INTRODUCTION

Colletotrichum lindemuthianum (Sacc. & Magnus) Briosi & Cavara, the causal agent of anthracnose in common bean (*Phaseolus vulgaris* L.), is one of the most yield-limiting factors for this crop in the world (Mahuku & Riascos, 2004; Schwartz et al., 2005). In the mountainous regions of South and Central America, severe anthracnose epidemics can lead to defoliation and reduce up to 95% of the crop yield when favorable weather conditions such as high humidity, moderate temperatures and high precipitation occur (Pastor-Corrales & Tu, 1989; Mahuku & Riascos, 2004; Schwartz et al., 2005). Bean anthracnose affects the quality of pods by inducing black, sunken cankers with a salmon-colored ooze centre containing millions of conidia (Melotto et al., 2000). Typical anthracnose symptoms such as the sunken lesions may also occur in petioles, leaf veins and stems (Melotto et al., 2000). In the case of infected bean seeds, they may have altered color but very often symptoms are absent, which constitute a problem when screening for disease-free material (Melotto et al., 2000; Schwartz et al. 2005). As this fungus is transmitted primarily by infected seeds and crop debris (Schwartz et al., 2005), the use of certified seeds is an important component in integrated disease management (Chen et al., 2013).

Traditionally, detection and identification of *C. lindemuthianum* has relied on its isolation using culture media and the observation of fungal morphological structures that requires relatively long incubation time (10-14 days) and experience of the analyst (Chen et al., 2007; 2013). Certification of disease-free donor plants and seeds depends on the availability of accurate, sensitive and time-effective diagnostic tools such as conventional and real-time PCR (qPCR), which has been proposed to detect *C. lindemuthianum* quickly and reliably using species-specific primers and or probes (Chen et al., 2007; 2013; Wang et al., 2008). DNA sequencing has also been used to differentiate closely related species within the main *Colletotrichum* clades (Cai et al., 2009; Cannon et al., 2012; Damm et al., 2013). The partial annotated genomes of *C. higginsianum* and *C. graminicola*, pathogens of maize (O'Connell et al., 2012) and of *C. orbiculare* and *C. gloeosporioides* isolated from strawberry (Gan et al., 2013) have identified pathogenicity-related genes.

Genomic regions most commonly used for the molecular characterization of fungi include ribosomal DNA (rDNA) and their internal transcribed spacers (ITS), mitochondrial DNA (mitDNA) (e.g. *Cox1*) and some protein genes such as actin (*ACT*), beta-tubulin (*TUB2*), calmodulin (*CAL*), chitin synthase I (*CHS-1*), manganese superoxide dismutase (*SOD2*) and the translation elongation factor 1-α (*EF1α*) (Hyde et al., 2009; Cannon et al., 2012; Liu et al., 2013). In *Colletotrichum*, species definition based on ITS sequence has proved unsatisfactory in some species-

complexes, so a multilocus genotyping approach combining ITS with several other genes or their introns, such as the 900-bp intron of the glutamine synthetase gene and the 200-bp intron of the glyceraldehyde-3-phosphate dehydrogenase gene, is used (Liu et al., 2007; Cannon et al., 2012).

Several primers have been designed for the detection of *C. lindemuthianum* (Chen et al., 2007; 2013; Liu et al., 2007; Wang et al., 2008); but their validity have been questioned and needs further confirmation because *C. lindemuthianum* has high levels of genetic variation (Apostolos et al., 2009; Pinto et al., 2012). In fact, *C. lindemuthianum* is a member of a clade of closely related species that originally included *C. orbiculare*, *C. trifolii*, and *C. malvarum* (Liu et al., 2007; Cannon et al., 2012). Based on spore morphology, appressorium development, and rDNA sequence similarities, it was proposed that these species should be regarded as a single species (Sherriff et al., 1994). Recently, Damm et al. (2013), studied the phylogenetic relationships of these fungi using a multilocus genotyping approach based on ITS, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), *CHS-1*, histone3 (*HIS3*), *ACT*, *TUB2* and glutamine synthase (*GS*) sequences of 42 strains of the *C. orbiculare* species complex. Their results confirmed the existence of the four species previously known as belonging to this species complex (*C. lindemuthianum*, *C. malvarum*, *C. orbiculare* and *C. trifolii*) and identified four new species: *C. bidentis*, *C. sidae*, *C. spinosum* and *C. tebeestii*.

Wang et al. (2008) developed a duplex PCR protocol for the detection of *C. lindemuthianum*. The first primer set, CY1/CY2, targets differences in the ITS region common in *C. lindemuthianum*. The second set of primers, CD1/CD2, was designed to amplify a DNA segment specific to *C. lindemuthianum* identified through RAPD markers. This primer amplifies a 638 bp segment and can distinguish *C. lindemuthianum* from *C. orbiculare*. Unfortunately, the sequence of the reported target region was not published and no further characterization of this region was performed. In this study, by sequencing the CD1/CD2 amplicon and 454 pyrosequencing of one *C. lindemuthianum* isolate, we demonstrate that the CD1/CD2 marker corresponds to a portion of an iron permease (*Ftr1*) pseudogene. The potential of using this genomic region for phylogenetic analysis of the Orbiculare clade *sensu* Cannon et al. (2012) and for the detection of their related species is discussed.

MATERIALS AND METHODS

Collection of isolates and DNA extraction

C. lindemuthianum isolates were obtained from leaves and pods of bean plants grown in the province of Antioquia (Colombia), var. Cargamanto showing anthracnose symptoms. They were collected at the municipalities of San Vicente Ferrer (04°34′51″N 74°08′20″W, 2150 MASL; isolates A51, A53 and A93), Sonsón (05°42′44″N 75°18′50″W, 2475 MASL; isolate A77), Santa Rosa de Osos (06°38′51″N 75°27′37″W, 2550 MASL; isolates A26, A37, A39, A76, and A90) and Urrao (006°18′55″N 76°08′03″W, 1800 MASL; isolates A47, A48 and A95). Isolate A83 from El Carmen de Viboral (06°05′06″N 75°20′19″W, 2150 MASL) previously identified as *C. lindemuthianum* was used as positive control for PCR. Samples were disinfected using 1% sodium hypochlorite for 1 minute followed by a second wash using sterile distilled water. Isolates were cultured in Potato Dextrose Agar (PDA) supplemented with both penicillin and tetracycline (100 mg/L) at room temperature (20ºC-24ºC) for 5-7 days, transferred to a new PDA medium without antibiotics and identified morphologically using light microscopy.

DNA was extracted from mycelia using the CTAB method of Doyle & Doyle (1990) with modifications from Santa et al. (2012). Mycelia were obtained from liquid cultures in 2% malt extract (ME) after 16-20 days of growth at room temperature in the dark. Three to five hundred mg of mycelia macerated in liquid nitrogen were used for each extraction. The integrity of the DNA was verified by agarose gel electrophoresis (0.8%) and purity determined by absorbance readings at 260 nm and 280 nm using a Nanodrop 2000C (Thermo).

PCR amplifications

The identity of each isolate was further confirmed by PCR amplification and sequencing of the rDNA ITS region using universal primers ITS1 (5’ TCC GTA GGT GAA CCT GCG G 3’) e ITS4 (5’ TCC TCC GCT TAT TGA TAT GC 3’) (White et al., 1990). Duplex PCR was performed following the procedure and conditions reported by Wang et al. (2008) using *C. lindemuthianum* specific primers CY1/CY2 (CY1: 5’-CTT TGT GAA CAT ACC TAA CC-3’; CY2: 5’-GGT TTT ACG GCA GGA GTG-3’) and CD1/CD2 (CD1: 5’-ACC TGG ACA CAT AAG TCA AAG-3’; CD2: 5’-CAA CAATGC CAG TAT CAG AG-3’). The size of the amplification products was confirmed using 1.8% agarose electrophoresis. Amplicons were purified using either the QIAquick PCR Purification or the QIAquick Gel Extraction kit (Qiagen). Sequencing of the PCR products was performed in both directions using the *Big Dye Terminator* kit (Applied Biosystems) in an ABI Prism 3730XL sequencer (Applied Biosystems) at Macrogen (South Korea).

Pyrosequencing

DNA of a single isolate (A83) was extracted using a Qiagen blood and tissue minikit following the manufacturer`s recommendations for fungi. The only modification was the mechanical lysis step using 3mm Tungsten Carbide Beads. Purified DNA was quantified using picogreen fluorescent dye and DNA integrity was assessed by agarose gel electrophoresis. For the genome sequencing, we followed the Whole Genome Shotgun strategy using the ROCHEs 454 FLX(+) technology at the Centro Nacional de Secuenciación Genómica-CNSG, Universidad de Antioquia

(Medellín, Colombia). One complete PTP was sequenced for this study; reads below 50 bases were excluded from analysis.

Data analysis

Genome de novo assembly was carried out with the Newbler assembler v2.6 using the flags: -m -cpu 40 –urt. The contig containing the CD1/CD2 amplicon was identified using a local BLASTN search against the *C. lindemuthianum* 454 assembly data. Contigs containing *Ftr1* homologs were identified by a similar procedure but using a TBLASTN search and a set of *Colletotrichum* FTR1 proteins as query. Gene annotation was done manually. Initially a BLASTX search against a non-redundant database at NCBI was performed. Intron positions were inferred by a multiple alignment of mRNA sequences encoding the closest *Ftr1* homologs from *Colletotrichum* for each gene variant. GC composition analyses were performed using a custom-made perl script that calculated the percent GC composition with a moving window of 100 bp. Sequence alignments were performed with MUSCLE (Edgar, 2004). Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011). Phylogenetic analysis relationship of sequences was inferred using the Neighbor-Joining method (Saitou & Nei, 1987) using Kimura's two parameter model (Kimura, 1980) with 1000 bootstrap replicates (Felsenstein, 1985).

RESULTS

The CY1/CY2 and CD1/CD2 specific primers for *C. lindemuthianum* were tested in 12 isolates identified at the basis of morphology and obtained at different bean-growing municipalities in the province of Antioquia, Colombia. As expected, two bands of 442 and 638 bp in size were observed after the duplex PCR in six isolates (Figure 1A). Amplification of the low molecular weight band, corresponding to the rDNA internal transcribed spacer, was not very consistent across the isolates, with repeated analyses revealing a failure to amplify in six samples, including the positive control (Figure 1A). This can probably be explained by polymorphisms at the primer binding sites in the Colombian *C. lindemuthianum* isolates not observed in the original ITS data (Wang et al., 2008).

The high molecular weight band was amplified in 11 isolates; the corresponding amplicon resulted in a stronger band compared to the ITS amplicon. For isolate A76, amplification failed for both primer sets, however, the duplex PCR was successful in the second trial (not shown). Sanger sequencing of the CD1/CD2 amplicon revealed complete conservation across isolates. With the expectation to find the CD1/CD2 target sequence in *C. lindemuthianum*, isolate A83 was chosen for a high-throughput 454 pyrosequencing run which resulted in a total of 1,483,861 reads which assembled into 12212 unique sequences totaling 61,396,412 bp of the *C. lindemuthianum* genome. Using the CD1/CD2 amplicon sequence as query, an identical segment was found in a 4,076 bp contig (C1027). The binding site of primer CD1 as located at positions 762-781 with one A/G mismatch at the 5' position; primer CD2, on the other hand, is 100% complementary to the 1,379-1,398 region of C1027 (Figure 1B). According to this analysis, the exact size for the CD1/CD2 amplicon must be 636 bp in the Colombian *C. lindemuthianum* isolates. A BLASTN search in the NCBI database resulted in a single hit to a 1,134,473 bp unplaced genomic scaffold of *C. orbiculare* MAFF 240422 (gb: KB726112) (Gan et al., 2013).

In order to verify if the CD1/CD2 amplicon corresponds to a protein coding region a BLASTX search was performed. Surprisingly, the CD1/CD2 sequence shared significant similarity with the C-terminal end of high-affinity iron permeases, also known as FTR1. Even at very low stringency, BLASTX failed to detect significant similarity to the N-terminal protein of the FTR1 proteins. A similar result was obtained for the corresponding region in *C. orbiculare* (not shown). Within *Colletotrichum,* the best scoring segment corresponds to residues 200-338 of a 366 a.a. FTR1 protein from C. *graminicola* (Figure 2A). A 15-residue deletion mapping with the third intron of the *C. graminicola* gene was found. Nucleotide changes at each codon position in the *C. lindemuthianum* pseudogene are shown in Table 1, comparing 417 nucleotide positions to their corresponding codon in *C. gloeosporioides* (gb:KB020675) and *C. graminicola* (gb:380495603). The former result, together with the presence of two stop codons in the translated aligned sequence, suggested the possibility that the CD1/CD2 region might be a *Ftr1* pseudogene. A search in the contig database from *C. lindemuthianum* revealed the existence of at least two intact *Ftr1* genes. The first *Ftr1* spans positions 15,346-16,736 of a 581,607 nt contig (C0002) and has as closest homolog an *Ftr1* gene for *C. orbiculare* MAFF 240422 (gb:ENH77797). The second *Ftr1* gene was found at positions 7,652-8,929 of contig 00366 (C00366: 24,549 bp) and is homologous to a different *Ftr1* paralog from *C. orbiculare* MAFF 240422 (gb:ENH86230).

Phylogenetic analysis of available FTR1 proteins from *Colletotrichum s*pecies revealed the existence of at least three distinct clusters (Figure 2B). Clusters I and III include FTR1 encoded by contigs 0002 and 366 respectively; however no protein homologs of cluster II were found in our *C. lindemuthianum* 454 database. Interestingly, iron permease EFQ33393 from *C. graminicola,* shown to be similar to the CD1/CD2 amplicon (Figure 2B) clustered within group II suggesting that this amplicon could be a remnant of an ancient group II *Ftr1* gene.

Genes encoding for group II FTR1 proteins in *C. gloeosporioides* Nara gc5, *C. gloeosporioides* cg-14, *C. graminicola* M1.001 and *C. higginsianum* are located 179-560 bp downstream of a gene encoding for a hydroxyproline-rich glycoprotein (HPRG) (Figure 3). Further analysis of contig 1027 revealed the presence of HPRG at approximately the same location; a similar gene map was also for *Ftr1*

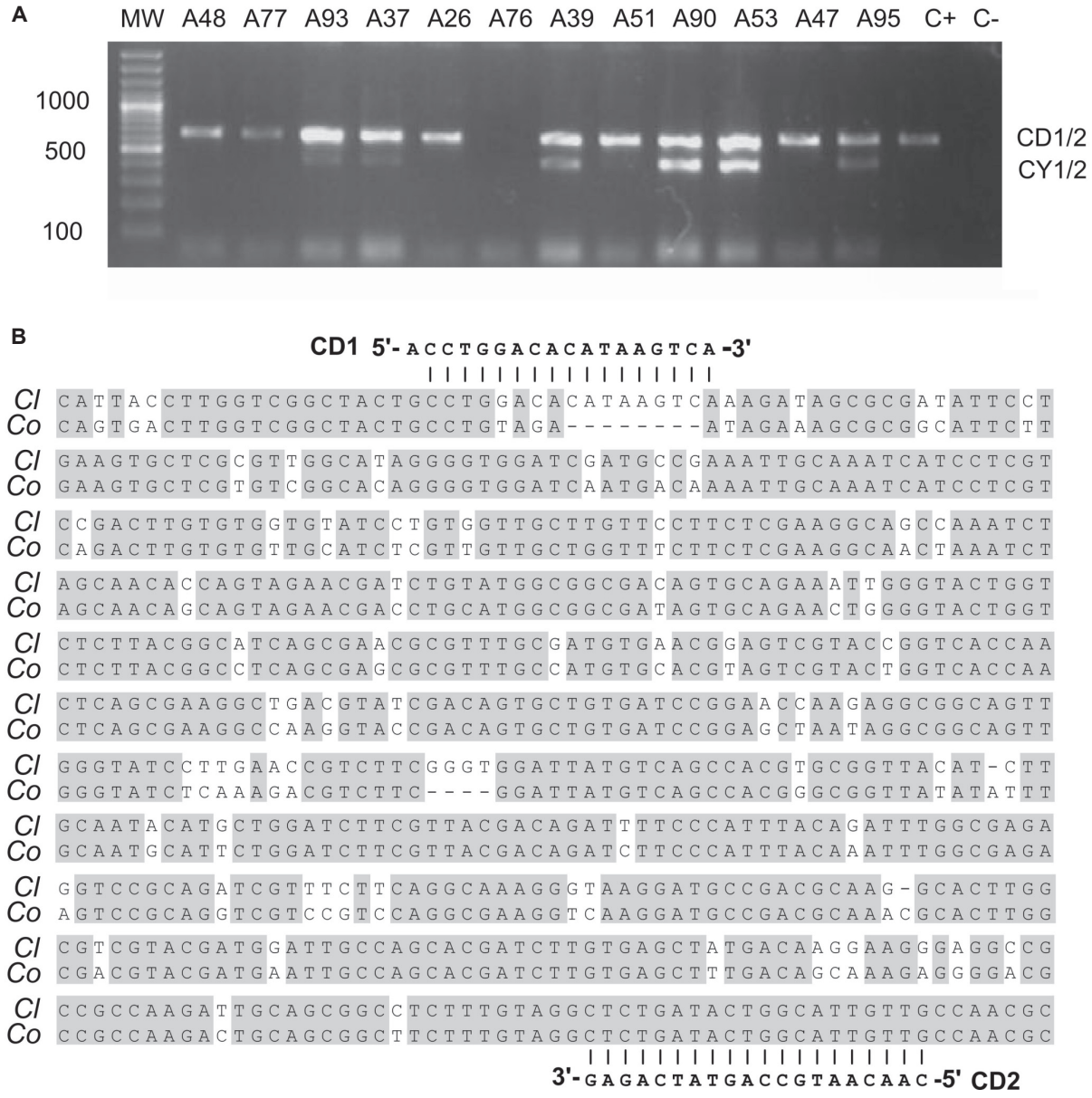

FIGURE 1 - Characterization of the CD1/CD2 amplicon in Colombian isolates of *C. lindemuthianum*. **A.** Duplex PCR using primers CY1/CY2 and CD1/CD2 in 12 *C. lindemuthianum* isolates from Colombia; **B.** Partial sequence of contig 1027 from *C. lindemuthianum* L87 (*Cl*) containing the target of primers CD1/CD2. The corresponding segment in *C. orbiculare* is aligned below (*Co).*

pseudogene in *C. orbiculare*. A side-by-side comparison clearly indicates that the N-terminal portion comprising the first two exons of group II *Ftr1* gene was lost in both *C. lindemuthianum* and *C. orbiculare* (Figure 3). This fact was further supported by sequence composition analysis, which shows that this region has a higher AT content as compared to the corresponding segments in the *Ftr1* pseudogen. The average GC content of the *Ftr1* genes, including their introns, varies from 55.4-55.5% in close agreement with the average GC values observed for the *C. lindemuthianum* (51.9%) and *C. orbiculare* (51.5%) pseudogenes. However, local GC composition changes dramatically to a GC poor region (15.2-18.7%) in the segment where the N-terminal portion of the *Ftr1* gene would be expected (Figure 3).

Phylogenetic analysis of the DNA segment comprising the CD1/CD2 region including all *Ftr1* known genes from *Colletotrichum* revealed that the *C. lindemuthianum* and *C. orbiculare* pseudogenes have a close relation to group II *Ftr1* (Figure 4). Predicted functional *Ftr1* genes identified in contigs 0002 and 00366 confirmed their higher similarity to groups III and I. Due to the degeneracy of the genetic code, functional coding sequences have a higher mutational

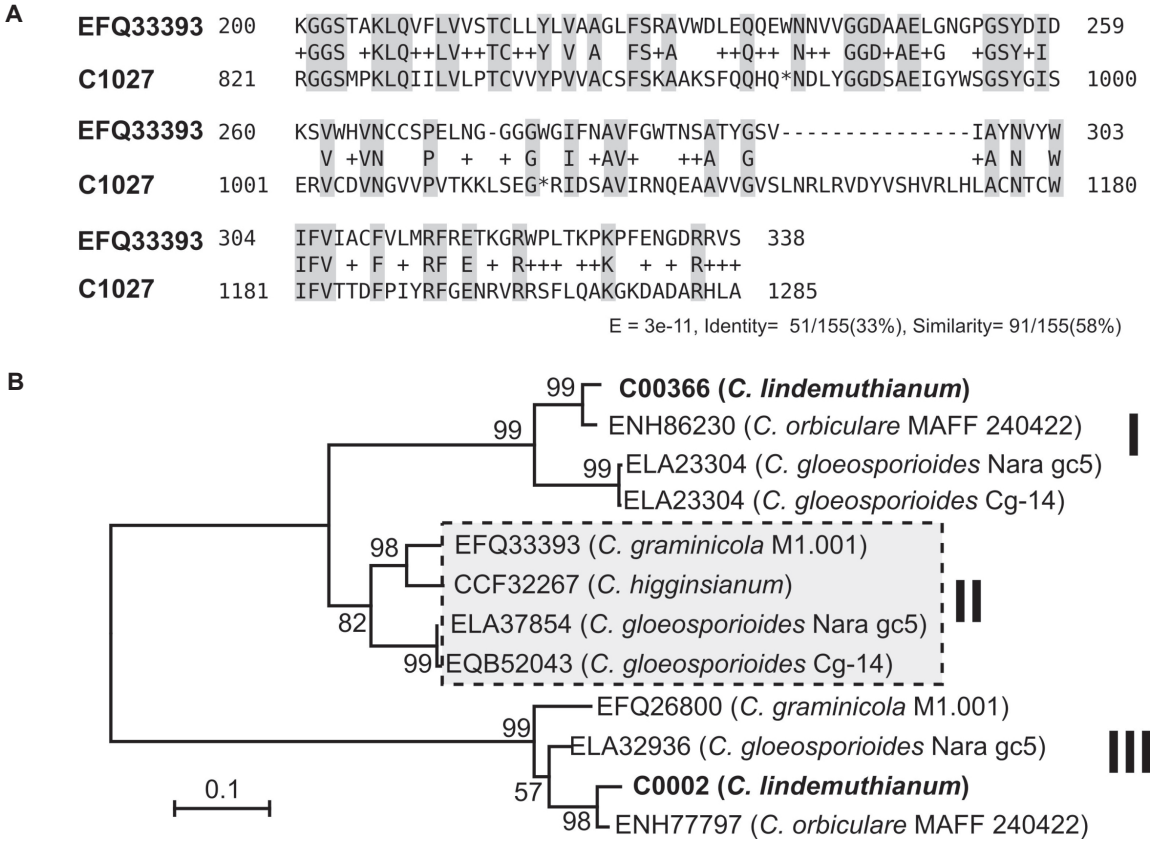

FIGURE 2 - Sequence analysis of translated CD1/CD2 amplicon. **A**. Alignment of the *C. graminicola* iron permease (EFQ33393) with the translated CD1/CD2 amplicon; **B.** Unrooted tree of iron permease protein sequences from *Colletotrichum* available in NCBI. C0002 and C00366 correspond to *C. lindemuthianum* proteins identified in this study.

TABLE 1 - Observed nucleotide changes at each codon position in the *Colletotrichum lindemuthianum* pseudogene. 417 nucleotide positions were analyzed and compared to their corresponding codon in *C. gloeosporioides* (gb:KB020675). The same analysis was performed on *C. graminicola* (gb:380495603, in parentheses).

Codon position	Transition		Transversion				Identical pair				n_d	p
	TC	AG	TA	TG	CA	CG	TT	CC	AA	GG		
First	**6**	**18**	**9**	**10**	**11**	**13**	**17**	**7**	**16**	**32**	**67**	**48.2**
	(2)	(5)	(1)	(4)	(2)	(3)	(27)	(18)	(30)	(47)	(17)	(12.2)
Second	**13**	**17**	**6**	**5**	**6**	**3**	**28**	**18**	**22**	**21**	**50**	**36.0**
	(3)	(2)	(1)	(2)	(1)	(2)	(38)	(25)	(33)	(32)	(11)	(7.9)
Third	**26**	**18**	**8**	**8**	**5**	**15**	**7**	**32**	**4**	**16**	**80**	**57.6**
	(27)	(13)	(3)	(4)	(9)	(11)	(8)	(40)	(3)	(21)	(67)	(48.2)
All	**45**	**53**	**23**	**23**	**22**	**31**	**45**	**57**	**22**	**69**	**197**	**47.2**
	(32)	(20)	(5)	(10)	(12)	(16)	(73)	(83)	(66)	(100)	(95)	(22.8)

n_d= Total number of substitutions; p, percentage of changes

rate at the third codon position as changes in these positions normally result in silent changes at the protein level. Analysis of the mutation rates of the *C. lindemuthianum* pseudogene revealed similar nucleotides changes at the first (48.2%) and second codon (36%) as in the third codon position (57.6%). This clearly contrasts with mutational rates observed in the functional *Ftr1* gene from *C. graminicola* where the mutation rate at the third position is much higher (48.2%) that the corresponding rates at position 1 (12.2%) and 2 (7.9%). As expected, the overall mutation rate in the

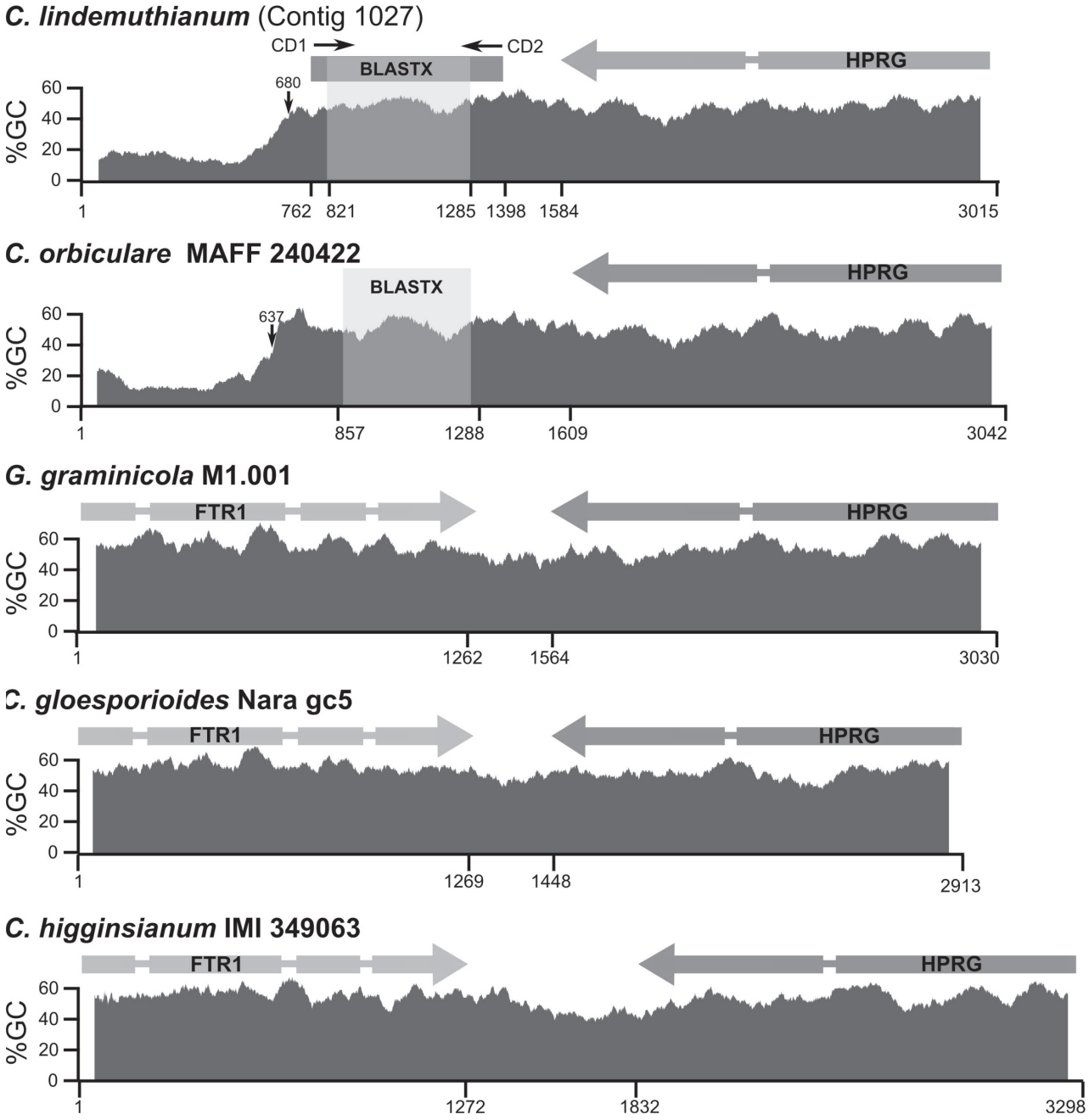

FIGURE 3 - Genetic map of the CD1/CD2 region in *Colletotrichum***.** *Ftr1* and *hprg* correspond to the gene position of the iron permeases and hydroxyproline rich glycoprotein; thin segments correspond to intron positions. The corresponding local GC content is plotted below and corresponds to the average of 100 residues surrounding the corresponding position. The compositional GC transition point in *C. lindemuthianum* and *C. orbiculare* is marked with an arrow; regions giving significant BLASTX results with FTR1 proteins are also indicated for these species. For *C. lindemuthianum*, the position of the CD1/CD2 is shown.

C. lindemuthianum pseudogene (47.2%) more than doubles the changes observed in *C. graminicola* (22.8%).

DISCUSSION

The availability of pathogen-free seed is key for the control of bean anthracnose (Schwartz et al., 2005). This requires highly sensitive and specific assays as those proposed by Wang et al. (2008) and Chen et al. (2013) based on duplex and qPCR, respectively. The use of molecular diagnosis, as a complement to traditional culture-dependent methods, in seed certification programs and quarantine vigilance of *C. lindemuthianum* would be very useful in the South American Andes where bean anthracnose is a major

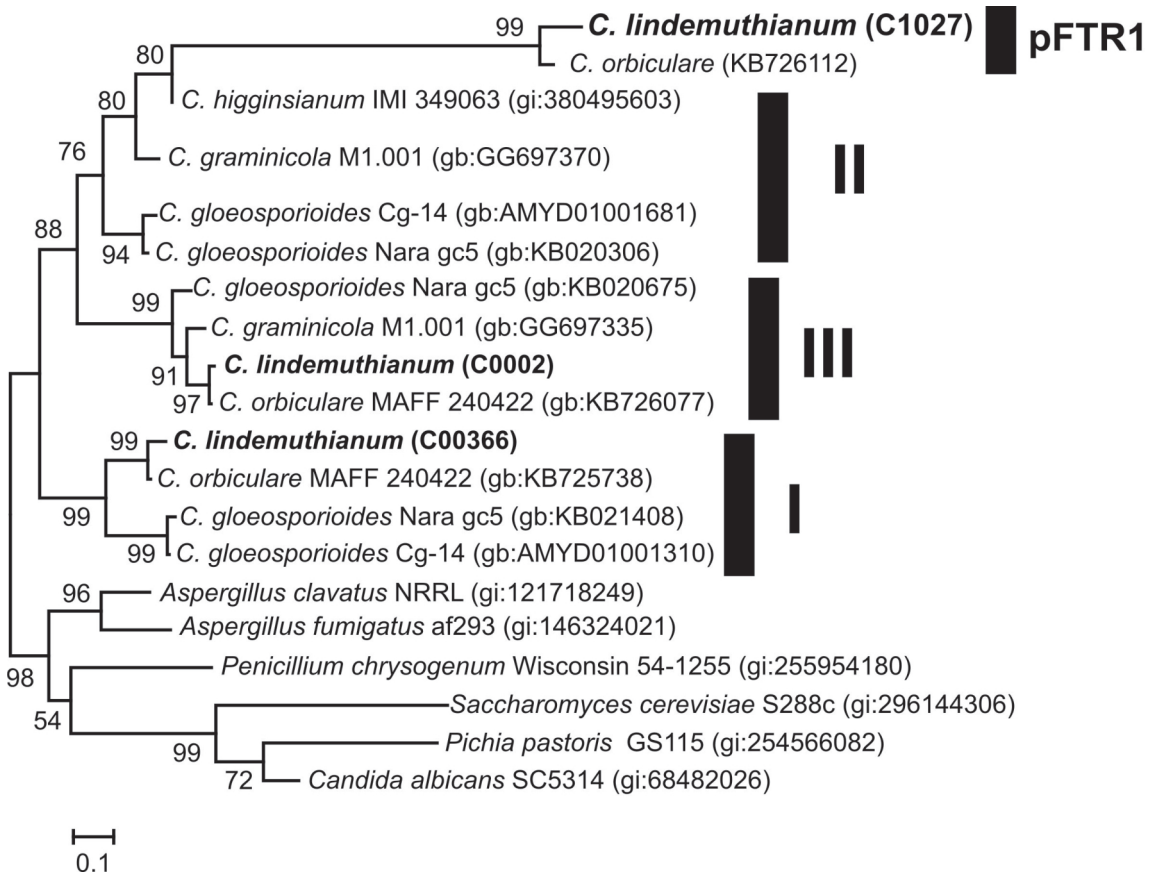

FIGURE 4 - Phylogenetic analysis of *Colletotrichum Ftr1* genes. *Ftr1* genes are divided into three main groups (I-III) in agreement with the corresponding protein analysis. *Ftr1* pseudogenes from *C. lindemuthianum* and *C. orbiculare* are more closely related to *Ftr1* genes in group II but show a much higher divergence rate as expected for a nonfunctional paralog.

disease (Pastor-Corrales & Tu, 1989; Mahuku & Riascos 2004). Unfortunately, our experience with the duplex PCR reported by Wang et al. (2008), using DNA from seeds, plant tissue (pods and leaves) and even *C. lindemuthianum* mycelia indicates that the CY1/CY2 amplicon may not be detected using standard PCR conditions. These primers were designed from a restricted set of 24 ITS sequences and captured the variability of this region of *C. lindemuthianum* and *C. orbiculare* while avoiding amplification from other *Colletotrichum* species and bean pathogens; however, it is likely that for some strains these primers fail to capture sequence variants from other geographic regions such as Colombia. By contrast, the CD1/CD2 primer set proved to be more reliable as it worked well for fungal detection in both symptomatic and asymptomatic seeds and plantlets. These primers were obtained by the random amplification of polymorphic DNA technique and were shown to be highly specific to *C. lindemuthianum* (Wang et al., 2008); unfortunately the authors did not report or characterized their target sequence. By sequencing the PCR product when using CD1/CD2 we showed that a pseudogene derived from the C-terminal end of a FTR1 high-affinity iron permease was identified in both *C. lindemuthianum* and *C. orbiculare*. This hypothesis is supported by the presence of stop codons, lack of exons 1 and 2, similar mutation rates at each position of the corresponding codons and mapping of this segment in the vicinity of the HPRG gene.

Sequence alignment clearly explained the specificity of the CD1/CD2 primer set; while primer CD2 is 100% complementary to its target region in both species, an 8 bp deletion in *C. orbiculare* disrupts binding of primer CD1 at the 3' end. From this result it was also evident that a new forward primer, analogous to CD1, can be designed to specifically identify *C. orbiculare*, adding up a new marker to identify this species to the sequence analysis of 900-bp intron of the glutamine synthetase gene used by Liu et al. (2007). Furthermore, each of the species in the Orbiculare clade (sensu Cannon et al., 2012) presented distinct host specificity: isolates of *C. orbiculare*, *C. lindemuthianum*, and *C. trifolii* being pathogenic only on cucurbits, common bean, and alfalfa, respectively, while de other species (*C. malvarum*, *C. bidentis*, *C. sidae*, *C. spinosum* and *C. tebeestii*) were found on different weeds (Damm et al., 2013).

FTR1 proteins, together with iron transport multicopper oxidases (fet3), are part of a conserved binary permease-oxidase complex involved in a high-affinity iron uptake in fungi (Philpott, 2006; Sutak et al., 2008). This system is essential for iron uptake in iron-limited environments and seems to be strongly related with pathogenesis in other fungal species such as *Candida albicans* and *Aspergillus oryzae* (Ramanan & Wang, 2000; Knight et al., 2002; Sutak et al., 2008). However, this system does not appear to be necessary for all fungi as neither *fet3* nor *Ftr1* has been identified in complete genome analysis of *Aspergillus nidulans* (Philpott, 2006). With the exception of C. *lindemuthianum* and *C. orbiculare*, three *Ftr1* paralogs where observed in all *Colletotrichum* species under study.

Little is known about the importance of these genes in the plant-pathogen interaction but it would not be surprising that they play a significant role in scavenging iron from their corresponding host during infection. In *Rhizopus oryzae*, the high affinity iron permease gene (*Ftr1*) is required for iron transport in iron-depleted environments and is required for full virulence in animal hosts (Ibrahim et al., 2010); a similar fact has been observed in the *Ustilago maydis*/maize pathosystem (Eichorn et al., 2006). Multiple *Ftr1* copies may help *Colletotrichum* species to have a broader range of possibilities to thrive in iron-poor environments. The lack of a functional *Ftr1* copy could explain the narrow host range of *C. lindemuthianum* and *C. orbiculare* in contrast with wide host-range pathogens such as *C. gloeosporioides*.

Eukaryotic pseudogenes are classified as processed or nonprocessed. Processed pseudogenes are retrotransposed into the genome via an RNA intermediate and therefore lack introns, may possess relics of a poly(A) tail and are often flanked by target-site duplications (Zheng et al., 2007). Nonprocessed pseudogenes, on the other hand, arise from non-homologous recombination events. The evidence presented here suggests that the *Ftr1* pseudogene of *C. lindemuthianum* is a nonprocessed pseudogene probably resulting from a non–homologous recombination event near the exon 2-exon 3 junction of the *Ftr1* gene. The long branch joining the *Ftr1* pseudogenes is expected as pseudogenes normally show higher divergence rate than functional genes and are subject of neutral degeneration right after their formation (Subramanian & Kumar, 2003). Loss of this gene probably had no major consequences as the presence of two additional paralogs could compensate for this gene loss. The similarity between the *Ftr1* pseudogene in *C. lindemuthianum* and *C. orbiculare* strongly suggests an evolutionary event that occurred previous to the separation of these closely related species (Liu et al., 2007; Cannon et al., 2012). The availability of the genomes of *C. lindemuthianum*, currently under study by our group, and *C. orbiculare* (Gan et al., 2013) will facilitate such evolutionary studies as well as a deeper understanding of key aspects of the host-pathogen interaction of this species complex. All these information may help to test the hypothesis of separation of these taxa from *Colletotrichum* at generic level due to its basal position in the phylogenectic studies developed for this genus (Cannon et al., 2012). Further work should explore the corresponding sequences in *C. trifolii* and *C. malvarum* to help clarify their close evolutionary relationship within the Orbiculare clade.

The discovery of CD1/CD2 *C. lindemuthianum* specific marker by Wang et al. (2008) together with the molecular characterization presented in this study opens the possibility of designing new markers based on iron permease genes and/or pseudogenes for detecting different *Colletotrichum* species and complement multilocus phylogenetic analysis for this important group of plant pathogens.

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