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Genetic markers for the analysis of variability and for production of specific diagnostic sequences in fumonisin-producing strains of *Fusarium verticillioides*

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Abstract

Fusarium verticillioides (*Gibberella moniliformis*, *Gibberella fujikuroi* mating population A) is the main source of fumonisins, a group of toxins which contaminates commodities, causing chronic and acute diseases in humans and animals. Fumonisins are produced during colonisation and infection of host plants even when disease symptoms are not recognisable. Early detection and control of *F. verticillioides* is crucial to prevent fumonisins from entering the food chain. DNA-based strategies have been used to search for markers to develop sensitive, robust and specific diagnostic assays, mainly based on PCR. The different approaches used, based either on DNA markers unrelated to fumonisin production or on information about the genes involved in fumonisin production, are described and discussed. The ability of these methods to discriminate between the two populations occurring within *F. verticillioides*, fumonisin-producing and fumonisin non-producing strains, is also addressed.

Introduction

Fumonisins are a group of structurally related polyketide-derivatives (Bezuidenhout et al., 1988) similar to the sphingoid base backbone of sphingolipids. Fumonisin B class includes the toxicologically important fumonisin B_1 (FB_1), FB_2 and FB_3, with FB_1 being the most abundant naturally occurring fumonisin, usually about 70–80% of total fumonisins produced in infected plants. They are produced by fungi belonging to *Gibberella fujikuroi* species complex (Leslie, 1995), among which *Fusarium verticillioides* (*G. moniliformis*, synonym *G. fujikuroi* mating population A) is the main source of fumonisin in cereals, particularly in maize and maize-based products.

Fumonisins cause several diseases in animals such as leukoencephalomalacia in horses (Marasas et al., 1988), pulmonary oedema in swine (Harrison et al., 1990) and hepatic cancer in rats (Nelson et al., 1993) and are considered to be Group 2B carcinogens by the International Agency for Research on Cancer (1993). At cellular level, FB_1 inhibits ceramide synthase, the enzyme responsible for the acylation of sphinganine in the biosynthetic pathway for sphingolipids, disturbing their metabolism (Wang et al., 1991; Abbas et al., 1994). This leads to the accumulation of sphingosine and a parallel decrease of ceramids, promoting DNA fragmentation, decreased viability, and loss of regulation of differentiation and apoptosis (Tolleson et al., 1999). Apoptosis (PCD, programmed cell death) is also observed when *Arabidopsis* plants are treated with FB_1, as well as the induction of other defence responses typical of plant–pathogen interactions (Asai et al., 2000). FB_1 is structurally related to AAL-toxin, a compound produced by *Alternaria alternata* f. sp. *lycopersici*, the cause of stem canker in tomato (Abbas et al., 1994; Wang et al., 1996). However, while the involvement of AAL in tomato disease seems to be confirmed (Brandwagt et al., 2002), the role of fumonisins in plant pathogenesis is not clear yet.

The importance of *F. verticillioides* as a causal agent of several diseases in cereals, and the production of fumonisins during the course of host infection, even when no visible symptoms are observed (Bacon and Hinton, 1996), emphasize the need to control both pathogen growth and the production of fumonisin. In order to develop efficient strategies to achieve these goals, it is crucial to have more information about population structure and variability of the species as well as the biosynthetic and regulatory pathways of mycotoxin production. This information could also be used to develop rapid, sensitive and specific assays to detect the presence of mycotoxigenic species or lineages in the early stages of infection and to prevent mycotoxins entering the food chain.

Two basic approaches can be followed to develop reliable genetic markers and diagnostic assays to detect *Fusarium* toxigenic species: (i) the use of nuclear genetic markers unrelated to toxin biosynthesis in combination with a phylogenetic analysis and (ii) the use of genes related to toxin production. The use of both strategies will be further addressed in the case of the *G. fujikuroi* fumonisin-producing species, and particularly *F. verticillioides*.

Genetic markers of nuclear genes unrelated to fumonisin production

The choice of suitable DNA-based markers depends on the level of variability expected among individuals. RAPDs and AFLPs are methods used to detect variability at the intraspecific level or among closely related species and have been used to discriminate between species or a group of species within the *G. fujikuroi* complex (Voigt et al., 1995; Amoah et al., 1996; Leissner et al., 1997; Schlacht et al., 1997; Benyon et al., 2000; Jiménez et al., 2000). Both techniques require further work to analyse the precise bands responsible for discrimination of the species or the group of species in order to develop specific diagnostic assays, based on PCR techniques. Furthermore, the application of the results of the above studies is limited because of the different taxonomical classifications used to identify the strains or because the sample cannot be considered sufficiently representative of the species. Other limitations are inherent in the technique, including the problems of reproducibility that occur with RAPDs and the costs and expertise needed for AFLP methods.

A combination of PCR–RFLP methods applied to variable regions of genes (introns) (Donaldson et al., 1995) or to the internal transcribed spacer (ITS) and intergenic spacer (IGS) of the rDNA units has been widely used (Edel et al., 1997; Guidot et al., 1999; Mirete et al., 2003) because it is simple and reproducible. Both the ITS and IGS regions are variable, with IGS being more variable and thus suitable for intraspecific analyses. The IGS region appears to have evolved more rapidly than ITS (Hillis and Dixon, 1991). This method, in combination with appropriate reference strains, can be useful to characterize new strains within a group of closely related species when morphological identification is not clear (Mirete et al., 2003) and to explore the level of variability present among the individuals of a particular sample.

There are few PCR-based detection methods for *F. verticillioides* or other species of the *G. fujikuroi* species complex. Murillo et al. (1998) developed primers for *F. moniliforme*, but these were not fully specific and showed some cross-reaction with closely related species (Möller et al., 1999). A recent publication reports the analysis of a set of primers based on *fum5* sequence to detect *F. verticillioides* (Bluhm et al., 2002). However, only a few strains of this species and related *Fusarium* species, particularly those within the *G. fujikuroi* species complex, were tested and these results need verification.

Phylogenetic analyses of the *G. fujikuroi* species complex using a multilocus approach suggest the existence of a complex situation, depending on the species considered. *Fusarium subglutinans* (*G. fujikuroi* mating population E) seems to consist of several lineages which are probably clonal (Steenkamp et al., 2002), while in the case of *F. verticillioides* (*G. fujikuroi* mating population A) there are two lineages, one sexual and another probably asexual (Mirete et al., this issue). These results indicate that the sample used to develop the diagnostic sequences must include representative strains of those lineages and of the level of variability within those lineages, although it is not possible to exclude the possibility that new lineages will be found. Similarly, several attempts to produce specific diagnostic sequences for the detection of trichothecene-producing *Fusarium* species have been published (Edwards et al., 2001), although some of them lack the support of a phylogenetic

analysis, produce cross-reactions with closely related species or do not include a reliable representative sample of strains (Edwards et al., 2002).

On the basis of the information obtained from the phylogenetic study of *F. verticillioides*, two sets of primers were developed based on the IGS sequence. Primers VERT-1 (5′GTCAGAATCCATGCCAGAACG3′) and VERT-2 (5′CACCCGCAGCAATCCATCAG3′) specifically detected strains of this species. The second set of primers, VERTF-1 (5′GCGGGAATTCAAAAGTGGCC3′) and VERTF-2 (5′GAGGGCGCGAAACGGATCGG3′) detected those *F. verticillioides* isolates which produced fumonisins (Patiño et al., 2004). Both sets of primers were used in the same PCR reaction and were tested on a large sample of *F. verticillioides* strains and with genomic DNA from pure cultures of other closely and distantly related *Fusarium* species. The results confirmed their specificity to *F. verticillioides* (Patiño et al., 2004). Since the IGS region is part of the multicopy rDNA unit, the sensitivity of a PCR assay using primers based on IGS sequence would be higher than using primers based on single-copy sequences. An estimation for visual detection of PCR products on agarose gels stained with ethidium bromide resulted in detection limits of between 1 and 10 pg of DNA template for ITS primers and between 1 and 10 ng of DNA template for single copy based primers (*fum5*) in *Fusarium* (Bluhm et al., 2002).

Genetic markers based on genes related to fumonisin biosynthesis

The structure of the fumonisins was published in 1988 (Bezuidenhout et al., 1988; Gelderblom et al., 1988) and since then considerable efforts have been made to elucidate the biosynthetic pathway leading to different fumonisins. The similarity of their basic structure to the sphingoid backbone of sphingolipids suggests that their biosynthesis might be related. Mutants of *F. verticillioides* with altered fumonisin products were related to three, apparently linked, loci (Desjardins et al., 1996; Proctor et al., 1999). The basic 18-carbon polyketide of fumonisin backbone is thought to be catalysed by a polyketide synthase (PKS) encoded by *fum5* (=*fum1*) gene (Proctor et al., 1999). Condensation of the 18-carbon polyketide, previously activated with CoA, with alanine is also proposed and this would be similar to the situation described for the AAL-toxin of *Alternaria alternate* f.sp. *lycopersici* (Shier et al., 1991). The analysis with mutants of the three linked genes identified by genetic analysis in *G. fujikuroi* mating population A (*F. verticillioides*) (*fum1*, *fum2* and *fum3*) suggested that FB_2 and FB_3 were the precursors of FB_1 (Desjardins et al., 1996). On the other hand, five clustered genes (*fum5*, *fum6*, *fum7*, *fum8* and *fum9*) were identified in a cosmid library with a probe obtained by PCR using degenerate primers based on the conserved amino acid sequence of the KS (β-ketoacyl synthase) domain of type I PKS (Proctor et al., 1999; Seo et al., 2001). Putative functions were assigned to the rest of the clustered genes on the basis of their sequence. Their expression pattern indicated that they were co-regulated in a way compatible with fumonisin detection in liquid cultures. The genes *fum5*, *fum6* and *fum8* were disrupted and the corresponding mutants produced little or no detectable fumonisins.

Recently, the analysis of the flanking regions of that cluster containing the five genes detected 18 additional putative genes (open reading frames, ORF) among which 10 were involved in fumonisin production (Proctor et al., 2003). The evidence provided was based on their co-expression with the five previously described genes and their similarity to other genes with functions compatible with fumonisin biosynthesis. Three of those 10 genes were disrupted: ORF-17 and ORF-18, both putative longevity assurance factors, and ORF-19, highly similar to ABC transporter genes. The results indicated that ORF-17 and ORF-18 disruptions did not affect fumonisin production, while the ORF-19 disrupted mutant showed a subtle effect on fumonisin production. This result suggests that this putative ABC transporter was not the only one involved in fumonisin transport and that other efflux pumps may work co-operatively to export fumonisin to the extracellular space. Evidence obtained in our laboratory indicates the participation of two additional genes encoding an ABC-transporter and a MFS-transporter (Mirete et al., 2002). The expression of these two genes was induced in liquid cultures of a strain of *F. verticillioides* producing FB_1 and FB_2.

Proteins involved in regulation have not been detected in the cluster, contrary to other mycotoxin biosynthetic clusters such as those which

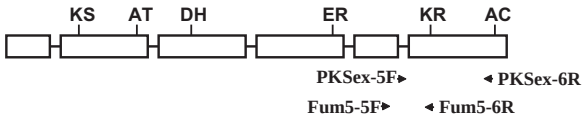

Figure 1. Location of the *fum5* gene domains: KS (β-ketoacyl synthase), AT (acyl transferase), DH (dehydratase), ER (enoyl reductase), KR (β-ketoacyl reductase) and AC (acyl carrier protein) and location of the primers designed for the detection of *fum5* gene (PKSex-5F/PKSex-6R and Fum5-5F/Fum5-6R).

synthesize aflatoxin or trichothecenes. Nitrogen and pH seem to affect the biosynthesis of fumonisins (Keller et al., 1997; Shim and Woloshuk, 1999), but there is little information about their regulation. Since fumonisins are secondary metabolites, it is expected that factors and regulatory networks involved in secondary metabolism and developmental clues would also have an effect in the regulation of their synthesis. One cyclin (FCC1) identified in *F. verticillioides* is apparently involved in both fungal development (conidiation) and regulation of fumonisin biosynthesis (Shim and Woloshuk, 2001). The identification and analysis of these factors will result in a better control of their synthesis under field conditions, thus reducing the impact of fumonisins to the food chain.

In summary, important information has been obtained about the biosynthesis of fumonisin and its genetic control, However, the biochemical pathway has not been fully elucidated and no enzymes of the pathway have been identified or characterised. Progress is expected once the putative genes involved in fumonisin production are thoroughly analysed and correspondence can be established for *fum1*, *fum2* and *fum3*. At present, *fum5* seems to correspond to *fum1* (Proctor et al., 2003) and *fum9* to *fum3* (Butchko et al., 2003).

PCR diagnostics based on *fum5* gene

Polyketide synthase (PKS) encoded by *fum5* is a multi-domain enzyme similar to other PKS enzymes (Proctor et al., 1999). The complex array of polyketide secondary metabolites, including pigments and toxins which occurs in *Fusarium* species, suggests that many PKS genes occur in the *G. fujikuroi* species complex (Yoder and Turgeon, 2001). PKS genes have differences in the functional domains present which affect their structure. For example, PKS encoded by *fum5* has six domains:

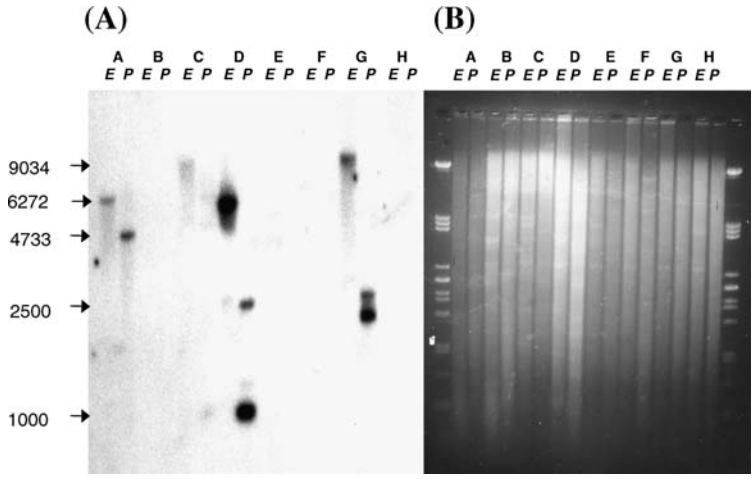

Figure 2. (A) Southern hybridisation with a *fum5* probe using moderately restricted conditions of genomic DNA of *F. verticillioides* (A), *F. sacchari* (B), *F. fujikuroi* (C), *F. proliferatum* (D), *F. subglutinans* (E), *F. thapsinum* (F), *F. nygamai* (G) and *F. circinatum* (H). Size of the hybridizing fragments is indicated in base pairs. (B) Total genomic DNA digested with the restriction enzymes *Eco* RI (E) and *Pst* I (P). λ: Genomic DNA from lambda phage digested with *Pst* I.

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                1                                                    60
MP-A            QCVAPYGKMLEIGKRDFIGKARLEMDLFEANRSFIGIDLARFDSARCQKLLERTAAMIQT
ITEM-2287       ...S.....................................S............VEA
MP-C            ..............L.........................................VEA
MP-G            ............................................................

               61                                                   120
MP-A            GIVQPIKPVKVFDASDAEGAFRYMQKGVHLGKIVVSIPPHSSTALPITPKPLQVKLNPEA
ITEM-2287       NSI.............-.................M..Q...L..VS.R.......A..
MP-C            NSI............S.E..................SQ...L..VS.R.......A..
MP-G            ..I.....................................V..V.........A..

              121                                                   180
MP-A            SYLLVGGLGGLGRAAATWMVESGARYLIFFSRSAGLSVRDQAFFQELASQGCTAQAVRGD
ITEM-2287       ..............T......R..................KT..R..............
MP-C            ...................R..................KT..R..............
MP-G            ..................................A.....................

              181                                                   240
MP-A            VLNLADVELAMASAPPGKPIRGVLQMSMVLRDKPFADMSLEDWDTAVKPKVHGTWNLHLA
ITEM-2287       ..S.P...RSV....A.....................................I...
MP-C            ..S.P...RSV....A.....................................
MP-G            ............................................................

              241                                                   300
MP-A            APKDLDFFFATGSISGSFGTPGQANYAAGNTYLTALFEHRRALGLPASVLQIGLIEDIGY
ITEM-2287       ............................................................
MP-C            ............................................................
MP-G            ............................................................

              301                                                   360
MP-A            LAKNPERAEALRAAGGFFLRTRQLLQGLNWALLSSDPHHPEYQLTIGLRSDKALSDPANR
ITEM-2287       ..Q..............-.....E.....................V....S.......
MP-C            ..Q.....................E.........................S.......
MP-G            ................................A.....................

              361     367
MP-A            VIWKKDS
ITEM-2287       .......
MP-C            .......
MP-G            .......
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Figure 3. Partial alignment of *fum5* gene in the *Gibberella fujikuroi* species complex: *F. verticillioides* (MP-A) showing the KR domain underlined. *F. proliferatum* (ITEM-2287) was supplied by A. Logrieco (ISPA, Bari, Italy), *F. fujikuroi* (MP-C) and *F. nygamai* (MP-G). Amino acid sequences were deduced from nucleotide sequences. The arrow indicates the position of the intron between exon 5 and exon 6. A dot represents the same amino acid residue as in the sequence of MP-A and a dash indicates that the amino acid in this position was not present.

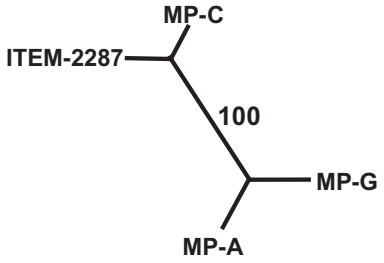

Figure 4. Phylogenetic tree of the partial *fum5* gene in the fumonisins-producing *Gibberella fujikuroi* species complex based on Jukes and Cantor distances with assigned bootstrap. *F. verticillioides* (MP-A), *F. proliferatum* (ITEM-2287), *F. fujikuroi* (MP-C) and *F. nygamai* (MP-G).

KS (β-ketoacyl synthase), AT (acyl transferase), DH (dehydratase), ER (enoyl reductase), KR (β-ketoacyl reductase) and AC (acyl carrier protein) (Figure 1). The KR domain is not present in other PKS genes involved in pigment biosynthesis (Linnemannstons et al., 2002).

The polyketide synthase gene can be used to design a diagnostic tool to detect fumonisin-producing strains and to quantify mRNA production of the gene through a real time PCR assay. The genes involved in trichothecene biosynthesis, particularly the *tri5* gene (trichodiene synthase) which catalyses the first step in the pathway (Desjardins et al., 1993), have been used to develop PCR assays to detect trichothecene-producing *Fusarium* species in fungal cultures and in cereal samples (Niessen and Vogel, 1998; Doohan et al., 1999; Edwards et al., 2001). A similar approach can be followed for fumonisin-producing species using

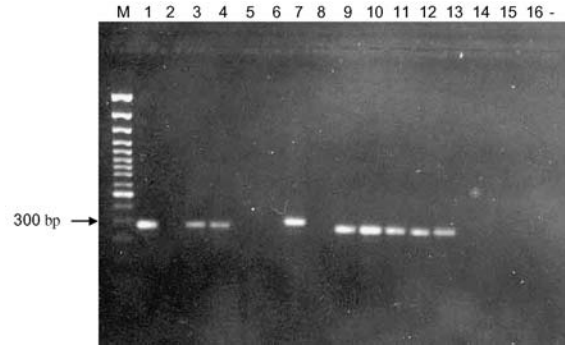

Figure 5. PCR amplification of a fragment of *fum5* gene using primers Fum5-5F/Fum5-6R in genomic DNA from *G. fujikuroi* mating populations A-H (lanes 1–8), fumonisin-producing strains of *F. verticillioides* (lanes 9–12), fumonisin-producing *F. proliferatum* strain (ITEM 2287) (lane 13) and fumonisin non-producing strains of *F. verticillioides* (lanes 14–16). M: DNA marker. (–): No DNA.

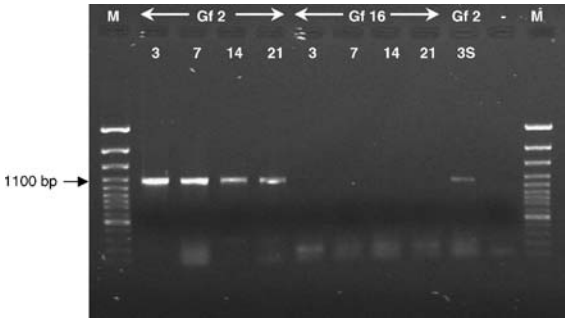

Figure 6. RT-PCR of *fum5* gene using primers PKSex-5F/PKSex-6R from cDNA from the fumonisin-producing *Fusarium verticillioides* strain Gf 2 and the fumonisin non-producing *Fusarium verticillioides* strain Gf 16 in fumonisin-inducing medium. (–) *F. verticillioides* genomic DNA; M: DNA marker. S: Gf 2 Sabouraud medium. The number indicates days of culture.

the *fum5* sequence to develop a PCR assay. Although production of fumonisins has been reported in many species outside the *G. fujikuroi* species complex (Rheeder et al., 2002), the species of this complex and, particularly, *F. verticillioides*, *F. proliferatum* (*G. fujikuroi* mating population D) and *F. nygamai* (*G. fujikuroi* mating population G) are the most important fumonisin producers that affect agricultural plant species. We have analysed the occurrence of some of the genes of the biosynthetic cluster, *fum5*, *fum6* and *fum8*, in the eight mating populations of the *G. fujikuroi* species complex: A (*F. verticillioides*), B (*F. sacchari*), C (*F. fujikuroi*), D (*F. proliferatum*), E (*F. subglutinans*), F (*F. thapsinum*), G (*F. nygamai*) and H (*F. circinatum*) by Southern blot hybridisation.

The presence of these genes was only detected in *F. verticillioides*, *F. proliferatum*, *F. fujikuroi* and *F. nygamai*. A Southern blot analysis corresponding to *fum5* is shown in Figure 2. A partial genomic sequence of *fum5* of these species was obtained and compared. The amplified region contained the KR domain. The alignment of the sequences is shown in Figure 3. Phylogenetic analysis revealed similar relationships (Figure 4) to these obtained with genes not related to fumonisin production (O'Donnell et al., 1998). Two specific primers were designed on the basis of these sequences: Fum5-5F (5′GAAATGGATCTA/CTTCGAGGC3′) and Fum5-6R (5′CCTTTCGATACATGCAGAAG 3′) (Figure 1). Both primers were used in a PCR assay with different *Fusarium*-related species, including strains from the fumonisin-producing and the fumonisin non-producing group of *F. verticillioides* (Figure 5). The lack of PCR product using fumonisin non-producing strains of *F. verticillioides* suggests they have lost the *fum5* gene or lost its function through an accumulation of mutations. However, no amplification occurred when primers based on *fum6* and *fum8* were used in PCR reactions, supporting the first hypothesis of a partial loss of the fumonisin cluster.

These primers and other sets of primers have been used to detect *fum5* expression by reverse transcription PCR assay. The assay was very sensitive and detected transcription when northern blot had not been able to, for example in Sabouraud medium, where fumonisin FB_1 was detectable (Figure 6). In this case, primers PKSex-5F (5′CGGTGTCAGAA-IntronV-ACTATTGGAA-CG3′) and PKSex-6R (5′GCACACGCGCTT CCCAATCTCAT3′) were used (Figure 1); PKSex-5F is an interrupted primer avoiding the problem of mRNA DNA contamination.

The identification of biosynthetic and regulatory genes will provide valuable information to develop tools for the detection of fumonisin at early stages. It should also lead to the control of fungal proliferation and toxin production in a selective way which could minimize the use of chemicals. Validated protocols (Martin et al., 2000) and integrated commercial kits will be the next step to make specific PCR assays useful and available to a wide community of users from field

to consumer, thus facilitating the goal of safer food and feed products.

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