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Nucleotide polymorphisms and protein structure changes in the *Fg16* gene of *Fusarium graminearum sensu stricto*

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ABSTRACT

Fusarium graminearum is one of the most important causes of wheat scab in different parts of the world. This fungus is able to produce widespread trichothecene mycotoxins such as nivalenol (NIV) and deoxynivalenol (DON) which are harmful for both human and animals. The *Fg16* target is located in chromosome 1 of the *F. graminearum* genome coding for a hypothetical protein whose function is not yet known. The *Fg16* gene is involved in lipid biosynthesis and leads to sexual development during colonization in wheat stalks. This gene is used to detect *F. graminearum* and determine the lineage of *F. graminearum* complex species. In the present study, polymerase chain reaction–single strand conformational polymorphism (PCR–SSCP) and DNA sequencing methods were employed in screening for genetic variation in 172 *F. graminearum s.s.* isolates. The PCR reaction forced the amplification of 410-bp fragments of *Fg16*. Two single nucleotide polymorphisms (T82C and A352T) and one amino acid exchange (C65S) with three patterns (TA/TA, CT/CT and TA/CT genotypes) were found in the *Fg16* gene fragment. Two haplotypes, 1A and 1B, were identified within *F. graminearum s.s.* populations in northern and western regions of Iran. Two different secondary structures of protein were predicted for CT/CT and TA/CT genotypes of *Fg16* gene. The average diversity levels detected were relatively high (He: 0.3238; He_u: 0.334; Ho: 0.2894; mean PIC: 0.514; mean Shannon's information index: 0.4132; mean number of alleles per locus: 1.473). On the basis of the obtained results, it was revealed that the *Fg16* gene had a high degree of polymorphism that can be considered for future control programming strategies and thus the associations between the SSCP patterns with different traits of *F. graminearum* such as wheat colonization, perithecium formation on stalk tissues and lineage discrimination should be investigated.

1. Introduction

Fusarium head blight (FHB) caused by *Fusarium graminearum* Schwabe [teleomorph *Gibberella zeae* (Schwein) Petch] is one of the most important fungal diseases of wheat worldwide that causes serious losses in both yield and quality of grain (Parry et al., 1995). This fungus produces different kinds of mycotoxins, which pose a serious health threat to humans and animals (Arseniuk et al., 1993). It has been estimated that 25% of the world food crops is affected by mycotoxins (Charmley et al., 1995). The most predominant mycotoxins found in small-grain cereals are 8-ketotrichothecenes (type B trichothecenes) such as deoxynivalenol (DON) (also known as vomitoxin) and nivalenol (NIV) and their acetylated derivatives including 3-acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON), as well as an estrogenic mycotoxin, zearalenone (Mirocha et al., 1989; Waalwijk et al., 2003).

Both sexual (ascospores) and asexual (macroconidia) propagules are produced by *F. graminearum* but ascospores are generally believed to be the primary inoculum of the disease (Shaner, 2003). Recent studies demonstrated that sexual development in *F. graminearum* is initiated during plant colonization (Guenther and Trail, 2005). The *Fg16* gene target is located at the XM381603 locus of chromosome 1 in the *F. graminearum* genome (http://www-genome.wi.mit.edu/annotation/fungi/fusarium/index.html) coding for a hypothetical protein (FG01427.1) whose function is not yet known.

Recently, it was found that the *Fg16* genes involved in lipid biosynthesis are highly expressed during vegetative growth and early sexual development in culture, and during colonization in wheat stalks (Guenther et al., 2009). Gene expression analysis suggests two phases of cellular growth with respect to lipid metabolism: a biosynthetic phase, where lipids are synthesized and accumulate, and a phase during which stored lipids are utilized to produce fruiting bodies. In *F. graminearum*, the *Fg16* gene, which is involved in mitochondrial lipid oxidation, shows expression typical of lipid oxidation genes (Guenther et al., 2009). Lipid stores are predominantly composed of triacylglycerides, which are characterized by a glycerol backbone and

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three fatty acyl side chains. The fatty acyl side chains vary (Murphy, 2001) and their composition tends to be species-specific (Weete, 1974).

Gene expression analysis during stages of perithecium development both in planta and in vitro supports the view that lipid biosynthesis occurs during early stages of wheat colonization leading to sexual development and that lipid oxidation occurs as perithecia are developing (Guenther and Trail, 2005). Analysis of gene expression during the stages of wheat stem colonization also revealed sets of genes unique to these stages and lipids accumulate in hyphae colonizing wheat.

Based on the *Fg16* gene, Fg16F/Fg16R primers were designed so that SCAR (sequence characterized amplified regions) types were determined accordingly. Carter et al. (2002) reported six SCAR types, based on PCR product size, among the FGSC isolates examined. These authors suggested that the SCAR product was characteristic for different groups within *F. graminearum sensu lato* (Carter et al., 2002). Polymerase chain reaction analysis specific to *F. graminearum* was performed using primer pair Fg16F/R, which produces polymorphic products with DNA from *F. graminearum* lineages, but no products with DNA from any other fungal species (Nicholson et al., 1998). The sequence of the Fg16F/R product is diagnostic of lineage and may be used simultaneously to detect *F. graminearum* and determine lineage (Carter et al., 2002; Nicholson et al., 1998).

It has recently been reported that *F. graminearum* consists of a number of lineages or groupings (Carter et al., 2000, 2002; O'Donnell et al., 2000). Analysis of a global collection of *F. graminearum* isolates from cereal hosts with primer set (Fg16F/ R) was found to produce one of six different PCR products from each isolate (Carter et al., 2002). Sequence analysis has revealed that the product of the Fg16F/R primer pair is diagnostic of the lineage/group and hence this primer pair may be used to detect *F. graminearum* and simultaneously determine lineage/group. Furthermore, because particular lineages/groups appear to be associated with geographic regions and mycotoxin chemotypes, the Fg16F/R assay can aid the detection of migrants and monitor pathogen movement.

SCAR analysis using Fg16F/Fg16R was previously used to distinguish *F. graminearum* and Fusarium asiaticum isolates from China (Qu et al., 2008). However, differentiation of *F. asiaticum* from Fusarium meridionale based on SCAR fragment size was not reliable. The use of SSCP analysis (single strand conformation polymorphism) enabled unequivocal resolution of *F. meriodionale* from *F. asiaticum*.

The objectives of the present study were the identification of *Fg16* gene mutations by PCR–SSCP and DNA sequencing methods and evaluation of the association between these mutations and protein structure changes in *F. graminearum sensu stricto* (*s.s.*) isolates.

2. Materials and methods

2.1. Sampling and Fusarium isolates

Diseased wheat spikes were taken during 2013–2014 from 245 fields in the northern and western regions of Iran that were separated by at least 10 km, and the numbers of the collected wheat spikes with clear FHB symptoms were proportional to the acreage of wheat infected by the disease. Each field was arbitrarily divided into 5 circular plots approximately 100 m in diameter, and 3–5 samples (spikes with visible infection symptoms) were randomly taken from each plot and then samples were pooled in each field. These collections represent samples from all the areas in the northern and western regions of Iran with a known history of FHB occurrences in wheat, covering 6 provinces including Golestan and Mazandaran (Northern regions), Hamedan, Kurdistan, Kermanshah and Lorestan (western regions).

After that the diseased wheat spikes were surface-sterilized and cultured in potato dextrose agar (PDA) and carnation leaf agar (CLA) media, *Fusarium* isolates were obtained by single spore isolation and identified by the methods described previously via Nelson et al. (1983). In total 172 isolates of *F. graminearum* were used in this study.

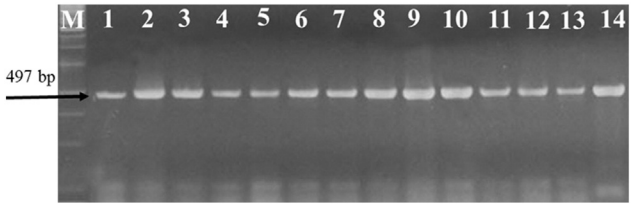

Fig. 1. PCR amplification products with Fg16F/Fg16R primer set. 1 to 14 isolates produce a 497 bp fragment. Product sizes for all isolates corresponded to SCAR type 1. M: marker(1 kb).

2.2. DNA extraction from fungal cultures

All isolated were inoculated with mycelia disks excised from the margin of 10-day-old PDA cultures in 100 ml Erlenmeyer flasks containing 20 ml of PDB liquid medium (Merck, Germany). Submerged fungi cultures were incubated on a rotary shaker at 120 rpm for 8 days at 25 °C. Mycelia were harvested by filtration through Whatman paper 1, ground to fine powder with liquid nitrogen and keep at −80 °C for further DNA extraction. Total genomic DNA was extracted from dried mycelium using the CTAB method as described by Nicholson et al. (1997).

2.3. SCAR and phylogenetic species analysis

SCAR analysis was used to identify the *Fusarium* isolates. It was previously demonstrated that SCAR typing, based on the size of the PCR product obtained with primer set Fg16F/Fg16R, resolved isolates of *F. graminearum* (type 1) and *F. asiaticum* (type 5) (Qu et al., 2008). A 410 bp DNA fragment specific for SCAR group I and a 497 bp fragment for SCAR group V were generated, respectively. Primer set Fg16F/Fg16R was used for SCAR analysis as described previously (Nicholson et al., 1998). Amplifications were carried out using 50 ng of DNA template with thermal cycling consisting of 30 cycles of denaturation (94 °C, 1.5 min), annealing (60 °C, 1 min) and extension (72 °C, 2 min). PCR products were separated on 2% agarose gels.

Portions of three phylogenetically informative genes (3-*O*-acetyltransferase (*Tri101*), translation elongation factor 1α (*EF-1α*) and reductase (*RED*)) from 10 isolates of *F. graminearum* were amplified and sequenced as described previously (O'Donnell et al., 2000, 2004). Lineage and phylogenetic species of *F. graminearum* were analysed and classified according to the methods of O'Donnell et al. (2000, 2004).

2.4. Single-strand conformation polymorphism (SSCP) analysis

The SSCP analysis of *Fg16* gene was carried out as follows. A 5 μL PCR product sample of BMPR-1B gene exon 8 was added to 10 μL of SSCP gel loading dye (0.05% bromophenol blue, 0.05% xylene cyanol, 95% formamide, 20 mM EDTA) and mixed. After heat denaturation at 97 °C for 8 min, the samples were immediately chilled on ice to prevent heteroduplex formation and then run (18 h, 300 V, 5 °C) on 15% acrylamide:bis-acrylamide gels (29:1 acrylamide to bisacrylamide), without glycerol in 1× TBE buffer on a 21 × 22 cm gel casting vertical electrophoresis (Payapajoohesh Pars, Iran). For SSCP analysis of Fg16, 4 μL of PCR sample was aliquoted into separate tubes, then 7 μL of SSCP gel loading dye was added and mixed. After heat denaturation at 98 °C for 10 min, the samples were immediately chilled on ice and then run (22 h, 300 V, 5 °C) on 8% acrylamide:bis-acrylamide gels (29:1 acrylamide to bisacrylamide), in 1× TBE buffer. DNA visualization in the polyacrylamide gel after electrophoresis was done by silver staining (Sanguinetti et al., 1994).