Optimization of a Specific Real-Time PCR Assay for *Fusarium avenaceum* Using Species-specific Couples of Primer

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ABSTRACT

Fusarium head blight (FHB) is an important cereal disease and may result in the accumulation of toxins in grains. Although *F. graminearum* is globally the most prevalent FHB causing species, *F. avenaceum* is found in contaminated samples. Real-time PCR (qPCR) is the the standard analytical method for species-specific, quantitative estimation of fungal biomass in the tissue of host organisms. qPCR is useful for quantifying fungal colonization of crops while distinguishing among species. Recently, species-specific PCR primers have been developed for most Fusarium species that cause head blight. In some cases, the species-specific primers synthesized with other *Fusarium* species and can give the wrong results. In this study, two species-specific primer pairs for *F. avenaceum* were tested by using with different fungi DNA. JIA primer pairs were amplified only *F. avenaceum*, whereas MGA primer pairs were amplified with *F. equiseti* and *F. tricinctum*. Results indicate that primer pair JIA is effective in determination of *F. avenaceum*.

Key words: *Fusarium avenaceum*, real-time PCR, wheat, species-specific primer

INTRODUCTION

Fusarium head blight (FHB), also known as scab or ear blight, is one of the most serious diseases in wheat growing areas (Parry et al. 1995; Goswami and Kistler 2004). FHB pathogens result in severe yield losses and a decline in cereal quality. Furthermore, infections by these pathogens lead to contamination of grain and straw by a wide array of mycotoxins. These fungal metabolites pose serious threats to human and animal health (Bennett and Klich 2003). FHB may be caused by 17 different Fusarium species, the most common of which are *Fusarium graminearum*, *F. culmorum*, *F. avenaceum*, *F. poae*, and *F. nivale* (Parry et al., 1995; Goswami and Kistler 2004). The predominant Fusarium head blight species besides *F. graminearum* are *F. culmorum F. avenaceum* and *F. poae*. *F. avenaceum* is widespread throughout the cool temperature cereal growwing areas and produces moniliformin, enniatins and fusarin (Desjardin, 2006; Yli-Mattila et al., 2006). Traditional diagnostic methods for detection and identification of *F. avenaceum* are based on micro- and macromorphological features developing on a medium. This process is time consuming and requires extensive training. In addition, it can often be difficult to distinguish between species having similar morphological characteristics. Recently, polymerase chain reaction (PCR) and Real-time quantitative-PCR are sensitive and rapid methods that can be used for the detection and screening of fungal microorganisms. Real-time PCR

(qPCR) is the standard analytical method for species-specific, quantitative estimation of fungal biomass in the tissue of host organisms. qPCR is useful for quantifying fungal colonization of crops while distinguishing among species. Real-time quantitative-PCR is a sensitive and rapid method that can be used for the detection and screening of *F. avenaceum*. More recently, real-time quantitative PCR assays with fluorogenic sequence-specific probes for *F. avenaceum* have been developed (Bluhm et al., 2004; Reischer et al., 2004; Waalwijk et al., 2004; Klemsdal et al., 2006; Yli-Mattila et al., 2006). Real-time PCR provides a means for detection and quantification of DNA targets by monitoring PCR product accumulation during the thermal cycling. The two main chemistries employed by real time-PCR are SYBR Green I and dual-labelled fluorescent probes, or TaqMan probes. Real-time PCR enables simpler and more rapid analysis of data, and has higher sensitivity and a wider dynamic range compared with conventional PCR (McKillip and Drake, 2004). Species-specific primers have been used for detection and screening of *F. avenaceum* (Schilling et al., 1996; Turner et al., 1998; Doohan et al., 1998; Waalwijk et al., 2003, 2004) and primers should be designed only for *F. avenaceum*. In some cases, the species-specific primers synthesized with other *Fusarium* species and can give the wrong results. The purpose of present study was to comparison of the different species-specific PCR primers for the identification of *F. avenaceum*.

MATERIALS AND METHODS

Fungal Isolates

The fungal strains used in this study are listed in Table 1. *Fusarium* spp. isolates for DNA isolation were grown in 100 ml of mungbean media with shaking (200 rpm) at 25 °C for 4 to 5 days. The mycelium was harvested by filtration and then freeze-dried. The other fungal isolates were grown in broth potato dextrose media.

DNA Extraction

A variant of the cetyltrimethylammonium bromide (CTAB) method described by Brandfass and Karlovsky, (2006) previously was used, and the quality and quantity of DNA were estimated by electrophoresis in 0.8% (w/v) agarose gel. The gel was stained with ethidium bromide (2 mg/lt) and documented with a digital imaging system (Vilber Lourmat, Marne la Vallee, France). The densitometry was performed using Multi-Analyst (Bio-Rad, Hercules, CA, USA). The concentration of fungal DNA was calculated by comparing a dilution series with defined amounts of DNA of lambda phage (methylated, from *Escherichia coli* host strain W3110).

Two couples of primer were used for *F. avenaceum*. One of them is JIAF (GCT AAT TCT TAA CTT ACT AGG GGC C) and JIAR (CTG TAA TAG GTT ATT TAC ATG GGC G) designed by Turner et al. 1998. The other one is MGB-F (CCATCGCCGTGGCTTTC) and MGB-R (CAAGCCCACAGACACG TTGT) designed by Waalwijk et al. 2004.

Real Time-PCR

The specificity of both PCR assays was determined with DNA extracted from pure cultures of 39 fungal isolates (35 *Fusarium* species and 4 isolates of other fungal species, Table 1). Real-time PCR was carried out in a total of 4 µl consisting of 10X buffer (Bioline, Germany), 2 mM $MgCl_2$, 150 µM dNTP (Bioline, Germany), 0.3 µM concentration of each primer, 0.025 u Taq DNA poymerase, (BIOTaq, Bioline, Germany), 0.1× SYBR

Green I (Invitrogen, Karlsruhe, Germany), and 1 µl template DNA. PCR reactions were performed in duplicate on all samples. Genomic DNA from pure cultures was diluted 1:100 before PCR.

Table 1. Fungal strains used in this study

Species	**Strain**	**Source**
F. subglutinans	Fsub2202	A
F. subglutinans	Fsub2215	A
F. subglutinans	Fsub2210	A
F. concolar	Fconc2	A
F. crookwellense	FCKW3	A
F. crookwellense	FCKW4	A
F. culmorum	FC15	E
F. culmorum	FC2	D
F. culmorum	FC22	E
F. culmorum	FCH69	A
F. graminearum	DSM6212	A
F. graminearum	DSM62722	A
F. graminearum	DSM67638	A
F. oxyporium	FO125	A
F. oxyporium	Foxy436	A
F. oxyporium	Foxy119	A
F. oxyporium	Foxy6	A
F. proliferatum	Fpro2	G
F. proliferatum	Fpro5	G
F. proliferatum	Fpro10	G
F. sacchari	Fsac1	A
F. sacchari	Fsac2	A
F. verticillioides	FRC8114	F
F. verticillioides	Fv	A
F. poea	*F. poe*	B
F. poea	*F. poe*	A
F. equiseti	*F. equiseti*	B
F. equiseti	*F. equiseti*	B
F. tricinctum	*F. trincinctum*	B
F. tricinctum	*F. trincinctum*	B
Septoria nodorum	SN1	A
Michrodochium nivale	GN7	E
Michrodochium nivale	GN35	E
Michrodochium nivale	GN36	E
F. avenaceum	Fa14	A
F. avenaceum	Fa16	C
F. avenaceum	Fa17	C
F. avenaceum	Fa18	A
F. avenaceum	Fa5-2	A

A: Culture Collection at the Göttingen University, the Division of Plant Pathology and Crop Protection, Germany; **B:** Marcel Tillmann, Göttingen University, the Division of Plant Pathology and Crop Protection, Germany; **C:** Berna Tunali, University of Ondokuz Mayis , Agricultural Faculty, Department of Plant Protection, Samsun, Turkey; **D:** H. Nirenberg (BBA Berlin, Germany) via E. Möller; **E:** T. Miedaner, State Plant Breeding Institute, University of Hohenheim, Stuttgart, Germany, via E. Möller; **F:** A. Desjardins, USA, Mexico, via E. Möller; **G:** A. Szecsi, Budapest, Hungary via E. Möller

PCR was performed on CFX384 Real-Time PCR Detection Systems (Bio-Rad, CA, USA). The amplification for *F. avenaceum* consisted of an initial denaturation at 94 °C for 2min, followed by 35 cycles of 30 s denaturation at 94 °C, 20 s annealing at 60 °C, and 30 s elongation at 72 °C. The final elongation step was performed for 5 min at 72 °C (Dastjerdi et al., unpublished).

RESULTS AND DISCUSSION

We investigated the species-specific PCR primers for *F. avenaceum* against 3 strains of *F. subglutinans*, one strain of *F. concolar*, two strains of *F. crookwellense*, four strains of *F. culmorum*, three strains of *F. graminearum*, four strains of *F. oxysporum* , three strains of *F. proliferatum*, two strains *F. sacchari*, two strain of *F. verticillioides*, two strains of *F. poe* , two strains of *F. equiseti* two strains of *F. tricinctum*, five strains of *F. avenaceum*, one strain of *Septoria nodorum* and three strains of *Michrodochium nivale*. Two couples of primer were evaluated for the specific real-time PCR assay for *F. avenaceum*. JIA and MGB which are specific for *F. avenaceum* were used to test *Fusarium* isolates. A standardized PCR protocol was established according to optimal conditions of all two primers (2mM $MgCl_2$, 200 µM dNTP, annealing temperature: 60 °C for 20 sec) (Dastjerdi et al., unpublished). Preliminary PCR assays, including melt curve analysis, were carried out with a BioRad iCycler (BioRad, Hercules, California, USA).

Primer couple of JIA specially amplified target DNA (*F. avenaceum*). All isolates of 14 non-target fungal species tested negatively. Samples of non-target fungal DNA generated no amplification products or unspecific products (Fig 1).

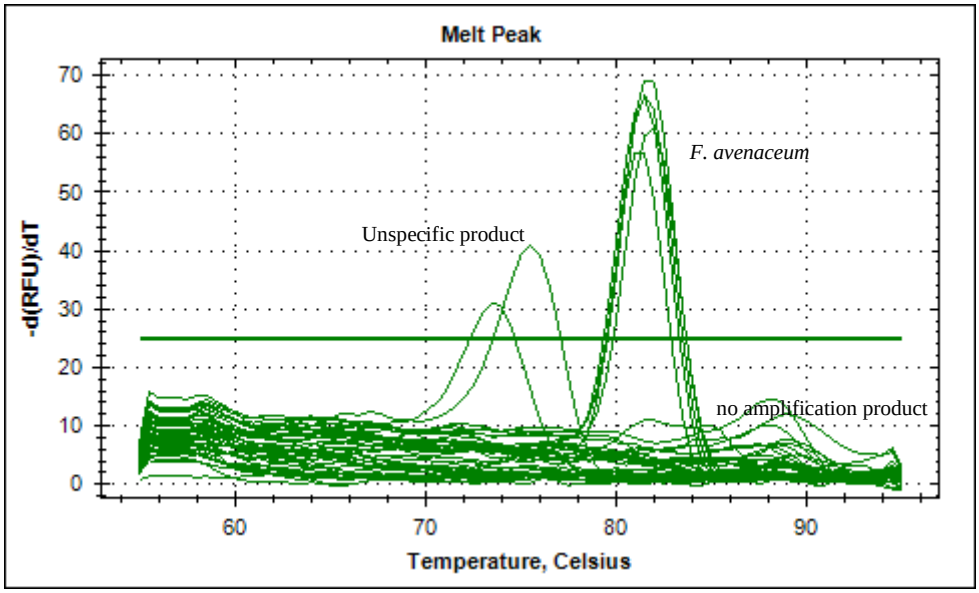

Fig 1: Melting curve analysis of PCR products obtained with primer JIA for *F. avenaceum*

Primer couple of MGB specially amplified target DNA (*F. avenaceum*). All isolates of 12 non-target fungal DNA generated no amplification products or unspecific products, but primer couple of MGB amplified *F. equiseti* and *F. tricinctum* (non-target DNA) with same melting temperature (Fig 2).

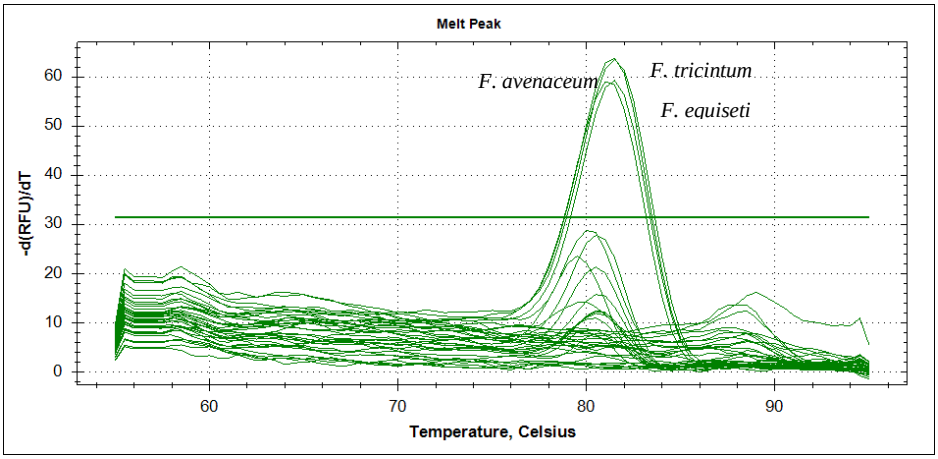

Fig 2: Melting curve analysis of PCR products obtained with primer MGA for *F. avenaceum*

Diagnostics for plant pathogens are important to identify fungal contaminants in crops and are invaluable for quarantine purposes. To confirm the morphological identification of species, PCR is performed by testing isolates with species-specific primers. Waalwijk et al 2002 tested seven primer pairs for their ability to identify isolates of *Fusarium avenaceum*, *F. culmorum*, *F. graminearum*, *F. poae*, *F. proliferatum* and *M. nivale* var. *majus* and *M. nivale* var. *nivale*. Each primer pair only generated a PCR product with the corresponding Fusarium species and all PCR fragments had different molecular sizes. Turner et al, 1998 tested the specificity of primers using primer pairs JIA for *F. avenaceum* against 2 strains of *F. graminearum*, two strains of *F. culmorum*, one strain of *F. poae*, one strain of *F. sporotrichioides*, thirty strains of *F. avenaceum*, one strain of *F. chlamydosporum*, one strain of *F. crookwellense*, one strain of *F. flocciferum*, one strain of *F. heterosporum*, four strains of *F. sambucinum*, one strain of *F. solani*, one strain of *F. sporotrichoides*, thirteen strains of *F. tricinctum*, and one or strains other fungal species. The primer was found to be specific for *F. avenaceum.* Yli-Mattila 2002 tested primer pairs JIA for *Fusarium avenaceum* against *Fusarium avenaceum/F. arthrosporioides/F. tricinctum* species. It was reported that JIA primer pairs was specific for *F. avenaceum*, but *F. avenaceum* was most similar to *F. tricinctum* isolates. In conclusion, primer pairs JIA, performed under real-time PCR conditions, generated positive signals only for the target species (*F. avenaceum*), while primer pairs MGB amplified non target DNA (*F. equiseti*, *F. tricinctum*). Primer pairs JIA can therefore be regarded as species-specific in real-time mode for *F. avenaceum*, respectively.

ÖZET

Fusarium avenaceum için tür-spesifik primerler kullanılarak real time PCR yönteminin optimizasyonu

Fusarium başak yanıklığı hastalığı tahıllarda çok önemli bir hastalık olup danelerde toksin oluşturmaktadır. Başak yanıklığı hastalığına dünyada en yaygın *F. graminearum* neden olmasına rağmen *F. avenaceum*'da hastalıklı örneklerde bulunmaktadır. Gerçek zamanlı PCR (qPCR) konukçu organizmaların dokusundaki fungal biyokütlenin, kantitatif olarak belirlenmesi için kullanılan standart analitik bir yöntemdir. qPCR türler arasındaki farklılığı belirlemede kullanıldığı gibi, kültür bitkileri içerisinde fungal kolonizasyon miktarının belirlenmesinde kullanılmaktadır. Son yıllarda başak yanıklığı hastalığına neden olan bir çok Fusarium türü için türe özgü primerler geliştirilmiştir. Bu çalışmada farklı funguslar ve *F. avenaceum* kullanılarak *F. avenaceum*'a özel iki primer (JIA ve MGA) test edilmiştir. JIA primer çifti sadece *F. avenaceum*'a bağlanırken, MGA primer çifti *F.equiseti* ve *F.tricinctum* ile bağlanarak çoğalmıştır. Sonuç olarak JIA primeri *F. avenaceum*'un tespitinde daha etkili bulunmuştur.

Anahtar kelimeler: *Fusarium avenaceum*, gerçek zamanlı PCR, buğday, tür-spesifik primer

LITERATURE CITED

Bennett, J.W., Klich, M. 2003. Mycotoxins. Clinical Microbiology Reviews 16:497–516.

Bluhm, B.H., Cousin, M.A., and Woloshuk, C.P. 2004. Multiplex real-time PCR detection of fumonisin-producing and trichothecene-producing groups of *Fusarium* species. Journal of Food Protection 67: 536–543.

Brandfass, C., and Karlovsky, P. 2006. Simultaneous detection of *Fusarium culmorum* and *F. graminearum* in plant material by duplex PCR with melting curve analysis. BMC Microbiology, 1-10 doi:10.1186/1471-2180-6-4.

Desjardins, A.E., Hohn, T.M., McCormick, S.P. 1993. Trichothecene biosynthesis in Fusariumspecies – chemistry, genetics, and significance. Microbiol Rev. 57:595–604.

Doohan, F.M., Parry, D.W., Jenkinson, P., and Nicholson, P. 1998. The use of species-specific PCR-based assays to analyse *Fusarium* ear blight of wheat. Plant Pathology 47:197–205.

Goswami, R.S., Kistler, H.C. 2004. Heading for disaster: *Fusarium graminearum* on cereal crops. Molecular Plant Pathology 5:515–525.

Klemsdal, S.S., Yli-Mattila, T., Paavanen-Huhtala, S., and Elen, O. 2006. Sensitive and quantitative real-time detection of trichothecene producing *Fusarium* spp. in cereal samples. European Journal of Plant Pathology (submitted).

McKillip, J.L., and Drake M.A. 2004. Real-time nucleic acid-based detection methods for pathogenic bacteria in food J. Food Prot., 67 (2004), pp. 823–832.

Nielsen, L.K., Jensen, J.D., Nielsen, G.C., Jensen, J.E., Spliid, N.H., Thomsen, I.K., Justesen, A.F., Collinge D.B., and Jorgensen, L.N., 2011. Fusarium head blight of cereals in Denmark: Species Complex and Related Mycotoxins, Population Biology,101(8):960-969.

Parry, D.W., Jenkinson, P., McLeod, L. 1995. *Fusarium* ear blight (scab) in small grain cereals - a review. Plant Pathology 44:207–238.

Reischer, G.H., Lemmens, M., Farnleitner, A., Adler, A., and Mach, R.L. 2004. Quantification of *Fusarium graminearum* in infected wheat by species-specific real-time PCR applying a TaqMan Probe. J. Microbiol. Methods 59, 141–146.

Schilling, A.G., Möller, E.M., and Geiger, H.H., 1996. Polymerase chain reaction-based assays for species-specific detection of *Fusarium culmorum*, *F. graminearum* and *F. avenaceum.* Phytopathology 86: 515-522.

Turner, A.S., Lees, A.K., Rezanoor, H.N., and Nicholson, P. 1998. Refinement of PCR-detection of *Fusarium avenaceum* and evidence from DNA marker studies for phenetic relatedness to *Fusarium tricinctum*. Plant Pathology 47:278–288.

Waalwijk, C., Kastelein, P., deVries, I., Kereny, Z., van der Lee, T., Hesselink, T. , Kohl, J.,and Kema, G. 2003.Major changes in *Fusarium* spp. in wheat in the Netherlands Eur. J. Plant Pathol., 109: 743–754.

Waalwijk, C., van der Heide, R., De vries, I., Van der Lee, T., Schoen, C., Costrel-de Corainville, G., Hauser-Hahn, I., Kastelein, P., Kohl, J., Lonnet, P., Demarquet, T., and Kema, G.H.J., 2004. Quantitative detection of *Fusarium* species in wheat using TaqMan. Eur. J. Plant Pathol. 110, 481–494.

Yli-Mattila, T., Paavanen-Huhtala, S., Parikka, P., Konstantinova, P., Gagkaeva, T., Eskola, M., Jestoi, M., and Rizzo, A. 2002. Occurrence of Fusarium fungi and their toxins in Finnish cereals in 1998 and 2000. J Appl Gen 43A:207–214.

Yli-Mattila, T., Paavanen-Huhtala, S., Parikka P., Konstantinova, P., and Gagkaeva, T. Y. 2004. Molecular and morphological diversity of *Fusarium* species in Finland and northwestern Russia. Eur. J. Plant Pathol.110:573-585.

Yli-Mattila, T., Paavanen-Huhtala, S., Parikka, P., Jestoi, M. Klemsdal, S., and Rizzo A.2006. Genetic variation, metabolites and mycotoxins of *Fusarium avenaceum* and related species. Mycotoxin Res., 22: 79–86.