Specific Detection and Identification of *Fusarium graminearum* Sensu Stricto Using a PCR-RFLP Tool and Specific Primers Targeting the Translational Elongation Factor 1 α Gene

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Abstract

Fusarium graminearum is a toxigenic plant pathogen that causes Fusarium head blight (FHB) disease on cereal crops. It has recently shown to have cross-pathogenicity on noncereals (i.e., Fusarium root rot [FRR] on soybean) in Canada and elsewhere. Specific detection and differentiation of this potent toxigenic, trichothecene-producing pathogen among other closely related species is extremely important for disease control and mycotoxin monitoring. Here, we designed a PCR restriction fragment length polymorphism protocol based on the DNA sequence of the translational elongation factor 1 α (*TEF1 α*) gene. A unique restriction site to the enzyme *HpaII* is only found in *F. graminearum* sensu stricto strains among different *Fusarium* strains in the *F. graminearum* species complex (FGSC) and other *Fusarium* spp. associated with FHB in cereals and FRR in soybean. Partial amplification of the *TEF1 α* gene with newly designed primers

mh1/mh2 generated a 459-bp PCR fragment. Restriction digestion of the generated fragments with the *HpaII* enzyme generated a unique restriction pattern that can rapidly and accurately differentiate *F. graminearum* sensu stricto among all other *Fusarium* spp. A primer pair (FgssF/FgssR) specific to *F. graminearum* sensu stricto also was designed and can distinguish *F. graminearum* sensu stricto from all other *Fusarium* spp. in the FGSC and other closely related *Fusarium* spp. involved in FHB and FRR. This finding will be very useful for the specific detection of *F. graminearum* sensu stricto for diagnostic purposes as well as for the accurate detection of this pathogen in breeding and other research purposes.

Keywords: detection, *Fusarium graminearum* species complex, PCR-RFLP, species-specific primers

Fusarium spp. are potent plant pathogens that cause several economically important diseases such as Fusarium head blight (FHB) and crown rot in cereals. *Fusarium graminearum* has been recently reported as a major necrotrophic pathogen of soybean causing seed decay, pre- and postemergence damping-off, pod blight, and Fusarium root rot (FRR) in Canada and other regions. Cross-pathogenicity of *F. graminearum* and other *Fusarium* spp. between cereal and soybean or pulse crops has been recently reported (Broders et al. 2007; Chiotta et al. 2016; Dill-Macky and Jones 2000; Sella et al. 2014; Xue et al. 2007). FHB in cereals is caused by several species of *Fusarium*, including *F. graminearum*, *F. avenaceum*, *F. cerealis*, *F. poae*, and *F. sporotrichioides*; however, *F. graminearum* is the primary species involved. FRR in soybean and pulse crops is caused by several *Fusarium* spp., most commonly by *F. oxysporum* and *F. solani* (Gray and Achenbach 1996; Roy 1997). Other *Fusarium* spp. have been reported as FRR-causative agents in soybean and pulse crops, including *F. redolens*, *F. acuminatum*, *F. chlamydosporum*, *F. culmorum*, *F. equiseti*, *F. incarnatum*, and *F. graminearum* (Bienapfl et al. 2010; Kulik et al.

2004; Lu et al. 2015; Zitnick-Anderson et al. 2018). Recently, there has been first report of the FHB pathogen *F. cerealis* causing root rot in soybean (Abdelmagid et al. 2018).

Some plant diseases can be managed by reducing the inoculum potential for future growing seasons. Inoculum can be reduced by eliminating the pathogen's spore-producing structures (Dill-Macky and Jones 2000) or removing the susceptible hosts species in a within-field crop rotation (Guo et al. 2010; Marburger et al. 2015). Crop rotation can be an effective and inexpensive method for managing some plant diseases but it is not necessarily effective for foliar or aerial diseases such as FHB in cereals. Nevertheless, because *Fusarium* spp. causing FHB (i.e., *F. cerealis*) were found to cause root rots in other crops such as soybean (Abdelmagid et al. 2018), rotation may contribute, at least in part, to FHB mitigation. Control of the disease is achieved by growing nonhost crops until the pathogen in the soil dies or its population decreases to a low enough level that there is negligible crop damage. Successful disease control by crop rotation requires complete understanding of the pathogen's life cycle. Some pathogens such as *Fusarium* spp. can survive in the soil for a long time even in the absence of its original host. *Fusarium* spp. can survive in the soil as a saprophyte on plant debris, and can also persist for many years as chlamydospores (Dill-Macky and Jones 2000; Lecomte et al. 2016; Marburger et al. 2015). Rotating wheat with soybean is one of the most frequently used crop rotation regimes in Canada, the United States, and other countries. Wheat offers a number of significant benefits when grown with soybean. It helps in controlling important pests and depletes the number of soybean cyst nematodes in the rhizosphere (Baird and Bernard 1984; Bajaj et al. 2015; Liebman and Dyck 1993; Schreiber 1992; Sindelar et al. 2016). *F. graminearum* is the most dominant *Fusarium* sp. that causes FHB in wheat and other cereal crops such as barley and oat around the world (McMullen et al. 1997). This pathogen can cause yield loss and grain contamination with trichothecene mycotoxins such as deoxynivalenol (DON) and its acetylated forms, 3ADON or 15ADON (Goswami and Kistler 2004; McMullen et al. 1997). *F. graminearum* was also shown to be a soybean pathogen causing seed decay, damping-off, and root rot (Broders et al. 2007; Ellis

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et al. 2011; Martinelli et al. 2004; Pioli et al. 2004). The transport of plant pathogens through the atmosphere involves three stages—liberation, drift, and deposition—and these stages are influenced by ecological and environmental factors (Schmale and Ross 2015). Abiotic factors (temperature, relative humidity, light, and wind) also play an important role in the spread of plant pathogens and the impact of these factors on the release of *F. graminearum* spores was previously investigated (David et al. 2014; Trail et al. 2002).

The *F. graminearum* species complex (FGSC) contains at least 16 phylogenetically distinct species (O'Donnell et al. 2000, 2008; Starkey et al. 2007; Yli-Mattila et al. 2009). It is extremely difficult to discriminate between members within the FGSC using morphological or microscopy-based methods. However, several molecular markers have been developed based on genetic differences for discerning species identity (Wang et al. 2011). Culture-based methods for identifying plant pathogens are time consuming and require extensive knowledge of classical taxonomy. Molecular methods have replaced the traditional phenotypic methods for accurate species identification, especially with morphologically and microscopically cryptic species such as *Fusarium* spp. Several PCR-based methods were developed for detection of species in the FGSC (Biazio et al. 2008; Jurado et al. 2005; Nicholson et al. 1998; Niessen 2007; Niessen and Vogel 1997; Schilling et al. 1996; Tan and Niessen 2003). These methods were designed to detect members in the FGSC (*F. graminearum* sensu lato) but none can specifically detect *F. graminearum* sensu stricto. Members of the FGSC are considered to be the main causative agent of FHB of wheat, barley, oat, and other small cereal grain crops worldwide. Many mycotoxins could be produced by FGSC, with trichothecenes being the most prevalent *Fusarium* mycotoxins in cereal grains; they are harmful to human and animal health, thereby posing a serious threat to food and feed safety (Aoki et al. 2012; Sudakin 2003). Trichothecenes are divided into four major groups based on their chemical structure: type A, type B, type C, and type D. Among these, type B trichothecenes are the most commonly detected mycotoxin in cereal grains and their related products (Placinta et al. 1999). Proper species identification is critical to any research aimed at improving disease and mycotoxins control. Accurate species identification for members of FGSC is highly important due to the ability of FGSC to produce many trichothecene mycotoxins, especially those from type B such as nivalenol (NIV) and DON, in addition to their acetylated derivatives 4ANIV, 3ADON, and 15ADON (van der Lee et al. 2015).

The Fg16F/R primer pair was originally designed to detect trichothecene-producing and nonproducing isolates of *F. graminearum* colonizing wheat grains (Nicholson et al. 1998). It targets the *Fg16* gene that is located in chromosome 1 in *F. graminearum* and other species in the FGSC, the gene codes for a hypothetical protein whose function is not yet known. Recently, it was found that there is a possible link between the *Fg16* gene and mitochondrial lipid oxidation (Guenther et al. 2009). Guenther and Trail (2005) studied gene expression during perithecial development both in planta and in vitro and they found that lipid oxidation occurs as perithecia are developing. Sequence-characterized amplified region (SCAR) analysis using the Fg16F/R primer pair was previously used to detect and differentiate between different species within the FGSC (Carter et al. 2002). However, differentiation of some species in the FGSC on the basis of SCAR fragment size was not reliable because this primer pair produces a polymorphic band for FGSC isolates *F. cortaderiae*, *F. brasilicum*, *F. meridionale*, *F. asiaticum*, *F. ussuriarum*, *F. louisianense*, and *F. mesoamericanum* (approximately 500 bp). *F. graminearum*, *F. austroamericanum*, *F. boothii*, *F. acaciae-mearnsii*, *F. nepalense*, *F. gerlachii*, *F. aethiopicum*, and *F. vorosii* produce amplicons smaller than 500 bp (Garmendia et al. 2018). *F. graminearum*, *F. nepalense*, *F. gerlachii*, and *F. aethiopicum* produce identical amplicons when amplified with Fg16F and Fg16R primers. Another FGSC-specific SCAR primer pair is UBC85F/R (Schilling et al. 1996), which produces monomorphic amplicons (332 bp) with all species in the FGSC. Neither the Fg16F/R nor UBC85F/R primer pairs are able to differentiate *F. graminearum* sensu stricto from other *Fusarium* spp. in the FGSC. Recently, a PCR restriction

fragment length polymorphism (RFLP) method was reported based on the translational elongation factor 1 α (*TEF1 α*) gene to differentiate *F. graminearum* sensu stricto from other species within the FGSC (Garmendia et al. 2018). This PCR-RFLP tool was designed to amplify the *TEF1 α* gene with the commonly used ef1/2 primer pair and digest the amplified PCR fragments with *Bsa*HI enzyme. The multiple recognition sites for *Bsa*HI (5'-GRCGYC-3'; GACGCC, GACGTC, GGCGCC, and GCGGTC) affect its specificity to *F. graminearum* sensu stricto and other *Fusarium* spp. (*F. asiaticum*, *F. pseudograminearum*, and *F. chlamydosporum*) and can be recognized and cleaved by the *Bsa*HI enzyme.

Several genes have been used to define species boundaries within the genus *Fusarium*: internal transcribed spacer (ITS) region (Edel et al. 1997; Schoch et al. 2012; Zarrin et al. 2016), mating type locus (Kashyap et al. 2015; Starkey et al. 2007), phosphate permease (Ortiz et al. 2017; Stakheev et al. 2016), trichothecene-3-O-acetyltransferase (Garvey et al. 2008; Kimura et al. 1998), and β -tubulin (β -TUB) (Mach et al. 2004). However, the *TEF1 α* gene has been the most frequently used sequence to define species boundaries within the genus *Fusarium* (Carbone and Kohn 1999; Garmendia et al. 2018; Geiser et al. 2004; Knutsen et al. 2004; Kristensen et al. 2005; Mbofung et al. 2007; O'Donnell et al. 1998, 2008; Zitnick-Anderson et al. 2018).

The objective of this work was to design molecular markers for specific detection of *F. graminearum* sensu stricto for rapid and accurate detection of this pathogen among other *Fusarium* spp. in the FGSC and other *Fusarium* spp. associated with FHB and FRR diseases in cereal crops and soybean, respectively.

Materials and Methods

Fungal cultures. *Fusarium* spp. isolated from several plant hosts (soybean, pea, wheat, barley, and oat) in Manitoba, Canada, were used in the current study. Wheat, barley, and oat *Fusarium* isolates were provided by Dr. Xiben Wang and Dr. Maria-Antonia Henriquez from Morden Research Center, Agriculture and Agri-Food Canada (AAFC, Morden, MB, Canada). Some soybean and pea *Fusarium* isolates were provided by Dr. Debra McLaren from Brandon Research Centre (AAFC, Brandon, MB, Canada). The remaining soybean *Fusarium* isolates as well as other plant pathogens such as *Colletotrichum spaethianum*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, and *Diaporthe caulivora* were collected and characterized by colleagues in our lab at the Plant Science Department, University of Manitoba, Canada. Nine species from the FGSC were obtained from the culture collection of United States Department of Agriculture–Agricultural Research Service (USDA-ARS, Peoria, IL, U.S.A.). The *Fusarium* isolates were initially identified based on morphological and microscopic characteristics and confirmed based on the *TEF1 α* gene sequence (see Table 1 for the complete species list).

DNA extraction and PCR amplification. Genomic DNA (gDNA) was extracted from single-spore cultures of each fungus using the GenElute plant gDNA miniprep kit (Sigma-Aldrich) based on the manufacturer's recommendations. The primer pairs ef1/ef2 and mh1/mh2 were used to amplify two fragments from the *TEF1 α* gene; primers and binding positions of these primers are shown in Figure 1 and Table 2. PCRs were performed in a final volume of 50 μ l using the DreamTaq Green PCR Master Mix (Thermo Fisher Scientific, Canada) with the following reagent concentrations: Green Taq buffer (1 $\times$), dNTP mixture (200 μ M each), forward and reverse primers (0.2 μ M each), Dream Taq DNA polymerase (1.5 U per 50 μ l), approximately 20:30 ng of gDNA template, and the total volume of the PCR adjusted to 50 μ l with nuclease-free H₂O. PCR amplification included an initial denaturation step at 94°C for 3 min; followed by 35 cycles of 94°C for 1 min, 50°C for 50 s, and 72°C for 1 min; and a final extension at 72°C for 5 min.

The Fg16 primer pair (Fg16F/R) was used for specific detection of the FGSC isolates. The PCR master mix was as previously mentioned above for the *TEF1 α* gene. The PCR amplification was performed according to Nicholson et al. (1998). FGSC-specific PCR detection with the UBC85F/R primer pair was performed according

to Schilling et al. (1996), with a PCR master mix as described for the *TEF1α* gene.

For specific detection of *F. graminearum* sensu stricto, two species-specific primers (FgssF and FgssR) were designed to have a 3' end unique to *F. graminearum* sensu stricto isolates exactly at the *HpaII* restriction site (Fig. 1). The FgssF primer was paired with ef2, while the FgssR primer was paired with FusF. The FusF primer is modified from the universal ef1 primer designed by O'Donnell et al. (1998) but with higher melting temperature (Tm) for optimum pairing with FgssR primer. These two primer pairs were used to detect *F. graminearum* sensu stricto and differentiate this pathogen among other *Fusarium* spp. as well as other pathogens associated with wheat and soybean. PCRs were performed in a final volume of 50 µl using the DreamTaq Green PCR Master Mix (Thermo Fisher Scientific, Canada) with the following reagent concentrations: Green Taq buffer (1×), dNTP mixture (200 µM each), forward and reverse primers (0.2 µM each), Dream Taq DNA polymerase (1.5 U per 50 µl), approximately 10 ng of gDNA template, and the total volume of the PCR adjusted to 50 µl with nuclease-free H₂O. Touchdown PCR protocol was utilized to insure primer specificity using an initial annealing temperature (Ta) higher than the optimal Tm of the primers and gradually reducing over subsequent cycles until the optimal Ta temperature was reached. PCR amplification included an initial denaturation step of 95°C for 3 min; followed by 1 min at 95°C, 30 s at Ta, and 1 min at 72°C; and a final extension for 5 min at 72°C. The initial Ta used with the FgssF/ef2 primer pair was 53°C for the first 5 cycles and 52°C for the next 5 cycles followed by 51°C for the last 25 cycles. The initial Ta used with the FusF/FgssR primer pair was 57°C for the first 5 cycles and 56°C for the next 5 cycles followed by 55°C for the last 25 cycles.

Specific detection of *F. graminearum* sensu stricto with FgssF/ef2 and FusF/FgssR primer pairs was also performed with Phusion high-fidelity (HF) DNA polymerase (Thermo Fisher Scientific, Canada). PCRs were performed in a final volume of 20 µl at the following reagent concentrations: 5× Phusion HF Green buffer (4 µl), 10 mM dNTP mixture (0.4 µl), 10 µM forward and reverse primers (1 µl each), Phusion HF DNA polymerase (0.2 µl) at 2 U/µl, approximately

10 ng of gDNA template, and the total volume of the PCR adjusted to 20 µl with nuclease-free H₂O. PCR amplification included an initial denaturation step at 98°C for 30 s; followed by 30 cycles of 98°C for 10 s, Ta for 15 s, and 72°C for 20 s; and a final extension at 72°C for 5 min. Ta for FgssF/ef2 was 59°C and for FusF/FgssR was 63°C.

Trichothecene genotyping based on *Tri12* multiplex PCR. Genotype determination of type-B trichothecene-producing *Fusarium* spp. was performed according to Ward et al. (2008) based on sequence diversity on the *Tri12* gene (encodes a trichothecene efflux pump). *Tri12* multiplex PCR produced amplicons of approximately 840, 670, and 410 bp with NIV, 15ADON, and 3ADON genotypes, respectively. PCRs for *Tri12* multiplex PCR were performed in a final volume of 25 µl using DreamTaq (Thermo Fisher Scientific, U.S.A.), with the following reagent concentrations: Taq green buffer (1×); dNTP mixture (200 µM each); 12CON, 12NF, 12-15F, and 12-3F primers (0.2 µM each); DreamTaq DNA polymerase (1.5 U per

Fig. 1. Schematic representation of the translational elongation factor 1α gene showing the exons (black boxes) and introns (gray boxes) and the binding sites of primers. The ef1/ef2 PCR fragment contains three *HpaII* restriction sites (5'-CCGG-3'); restriction sites 1 and 3 (gray triangles) are found in all investigated *Fusarium* spp., whereas restriction site 2 (black triangle) is found only in *Fusarium graminearum* sensu stricto and absent in all other *Fusarium* spp., including the *F. graminearum* species complex. The *HpaII* restriction site is found in intron number 2: 199 nucleotides from the mh1 primer and 260 nucleotides from the mh2 primer.

Table 1. List of *Fusarium* spp. used during the current study, along with translational elongation factor 1α (*TEF1α*) GenBank accession numbers, plant host, and country of origin^a

Species	Isolate	<i>TEF1α</i> accession	Host	Country	FGSC	Type-B
<i>Fusarium oxysporum</i>	Carm31	MH315926	Soybean	Canada	No	No
<i>F. redolens</i>	MLT5	MH315933	Soybean	Canada	No	No
<i>F. avenaceum</i>	HSW15.93	MH315905	Wheat	Canada	No	No
<i>F. acuminatum</i>	Carm2	MH315914	Soybean	Canada	No	No
<i>F. equiseti</i>	Carm35	MH315930	Soybean	Canada	No	No
<i>F. incarnatum</i>	Carm37	MH479893	Soybean	Canada	No	No
<i>F. poae</i>	MRC99	MH315911	Oat	Canada	No	No
<i>F. culmorum</i>	MLT10	MH315937	Soybean	Canada	No	3ADON
<i>F. cerealis</i>	Carm17	MH151080	Soybean	Canada	No	NIV
<i>F. cerealis</i>	Carm20	MH151081	Soybean	Canada	No	NIV
<i>F. graminearum</i>	HSW15.16	MH315907	Wheat	Canada	Yes	3ADON
<i>F. graminearum</i>	MRC85	MH315909	Oat	Canada	Yes	3ADON
<i>F. graminearum</i>	MRC31	MH315910	Oat	Canada	Yes	15ADON
<i>F. graminearum</i>	Carm3	MH315915	Soybean	Canada	Yes	15ADON
<i>F. graminearum</i>	Carm10	MH315922	Soybean	Canada	Yes	15ADON
<i>F. mesoamericanum</i>	NRRL 25797	AF212441	Banana	Honduras	Yes	NIV
<i>F. acaciae-mearnsii</i>	NRRL 26752	AF212447	<i>Acacia mearnsii</i>	South Africa	Yes	NIV
<i>F. aethiopicum</i>	NRRL 46710	FJ240295	Wheat	Ethiopia	Yes	15ADON
<i>F. boothii</i>	NRRL 26916	GQ915503	Corn	South Africa	Yes	15ADON
<i>F. brasiliense</i>	NRRL 31281	AY452964	Oat	Brazil	Yes	3ADON
<i>F. gerlachii</i>	NRRL 36905	DQ459742	Orchard grass	New Zealand	Yes	NIV
<i>F. meridionale</i>	NRRL 28436	AF212435	Orange twig	New Caledonia	Yes	NIV
<i>F. nepalense</i>	NRRL 54220	KM889629	Rice	Nepal	Yes	15ADON
<i>F. vorosii</i>	NRRL 37605	DQ459745	Wheat	Hungary	Yes	15ADON

^a *Fusarium* spp. belonging to *F. graminearum* species complex (FGSC) are indicated. Type-B trichothecene (Type-B) genotype based on *Tri12* multiplex PCR (nivalenol [NIV], 3-acetyl-deoxynivalenol [3ADON], or 15-acetyl-deoxynivalenol [15ADON]) for *Fusarium* spp. used in the current study are also indicated. *F. pseudograminearum* NRRL40886 was also tested during the current study.

50 µl); approximately 20:30 ng of gDNA template; and the total volume of the PCR adjusted to 25 µl with nuclease-free H₂O. Detailed information about the primers used in *Tri12* multiplex PCR is shown in Table 2. PCR amplification conditions for *Tri12* multiplex PCR included an initial denaturation step at 94°C for 2 min; followed by 30 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 1 min; and a final extension at 72°C for 5 min.

All PCR amplicons were analyzed by gel electrophoresis through 1 to 2% agarose gels in 1× Tris-borate-EDTA (TBE) buffer (89 mM Tris-borate and 10 mM EDTA, pH 8.0). The sizes of the PCR amplicons were estimated against a 1-kb-plus DNA ladder (Thermo Fisher Scientific, Canada) and visualized under UV light after staining with ethidium bromide (5 µg/ml). PCR products were purified with the Wizard SV Gel and PCR Clean-Up System (Promega, Canada) and sent for sequencing (Macrogen, Rockville, MD, U.S.A.).

Sequence analysis. Individual *TEF1α* gene sequences were compiled and assembled manually into contigs using the GeneDoc program, v2.5.010 (Nicholas et al. 1997). The initial nucleotide sequence alignments were done with the Clustal-X program (Thompson et al. 1997) and the resulting alignments were refined by eye with the GeneDoc program, v2.5.010. The online resource Basic Local Alignment Search Tool (<https://www.ncbi.nlm.nih.gov/BLAST/>) (Altschul et al. 1990) was used to retrieve similar sequences from the NCBI database to have significant representatives from each species investigated in the current study as well as sequences that represent other *Fusarium* spp. involved in FHB and FRR diseases. A phylogenetic tree was constructed using the iTOL v3 online tool (Letunic and Bork 2016) based on *TEF1α* sequences (see Supplementary Table S1 for GenBank accession numbers of *TEF1α* sequences used in the tree).

PCR-RFLP based on *TEF1α* gene sequence. *TEF1α* sequences for each *Fusarium* spp. were analyzed separately with the Restriction-Mapper program (version 3; <http://www.restrictionmapper.org/>). The *HpaII* restriction enzyme was chosen because it provided species-specific restriction pattern for *F. graminearum* sensu stricto. Based on sequence alignment for the *TEF1α* gene, the restriction site for *HpaII* (5'-CCGG-3') was found in all tested *Fusarium* spp. at two positions and found in *F. graminearum* sensu stricto at three positions (Fig. 1). A new primer pair, mh1/mh2, that binds conserved sequences in all *Fusarium* spp. was designed to amplify a fragment from the *TEF1α* gene in all *Fusarium* spp. This mh1/mh2 PCR fragment containing the *HpaII* restriction site is only found in *F. graminearum* sensu stricto and not in the FGSC strains or other *Fusarium* strains tested. Restriction digestion of the mh1/mh2 fragment was selected to give a unique pattern for all *F. graminearum* sensu stricto isolates, while any other *Fusarium* spp. DNA in that region will not be cut by the *HpaII* enzyme and gives a different restriction pattern.

Restriction digestions for mh1/mh2-*TEF1α* PCR amplicons with *HpaII* enzyme were performed with approximately 400 ng of the mh1/mh2 PCR products (10 µl) in a total volume of 30 µl, with 10× Tango buffer (2 µl) and 10 U of *HpaII* (Thermo Fisher Scientific, Canada) at 37°C. Time needed for complete digestion of the mh1/mh2 PCR amplicons by *HpaII* was tested by incubating the restriction mixture (described above) for 5, 10, 20, 30, and 60 min. Restriction digestions for mh1/mh2-*TEF1α* PCR amplicons was also performed using FastDigest *HpaII* enzyme (Thermo Fisher Scientific, Canada) with approximately 400 ng of the mh1/mh2 PCR products (10 µl) in 10× FastDigest Green buffer (2 µl), and 2 µl of FastDigest *HpaII* (Thermo Fisher Scientific, Canada), with a total volume adjusted to 30 µl with nuclease-free water for 5 min at 37°C. The resulting fragments were separated by gel electrophoresis (2% agarose) for 90 min at 95 V in 1× TBE (pH8.2). The gels were stained with ethidium bromide and visualized on the UV transilluminator. The size of digested bands was determined by comparing them with a 1-kb-plus DNA ladder (Thermo Fisher Scientific, Canada).

In planta experiments. An important objective during this study was to test the newly developed PCR-RFLP marker to specifically detect *F. graminearum* sensu stricto in infected wheat and soybean plant tissues. *F. graminearum* sensu stricto (isolate Carm3) and *F. cerealis* (isolate Carm1) were used to inoculate wheat and soybean to produce FHB and FRR, respectively. Wheat seed (Muchmore) were planted in 15-cm pots filled with sterilized ready-mix soil. They were grown in a greenhouse at approximately 20 ± 3°C with 16 h of supplemental light until flowering and anthesis formation. A signal central floret of the spikelet of the main stem was inoculated at anthesis with 10 µl of macroconidial spore suspension (5 × 10⁴ spores/ml) of *F. graminearum* sensu stricto Carm3 or *F. cerealis* Carm1 inoculated individually. Control plants were inoculated with 10 µl of sterile water. The spikes were covered with humid plastic bags for conidia germination and removed 36 h postinoculation. Plants were watered on odd days and treated with 20:20:20 nitrogen-phosphorus-potassium fertilizer and grown to full maturity. FHB symptoms were scored (nine spikes for each treatment) on a 0-to-100% scale, as described by Engle et al. (2003).

Soybean seed (22-60RY) were planted in ready-mix soil spiked with cornmeal (20 g/kg of soil) previously inoculated with a macroconidial suspension (5 × 10⁴ spores/ml) of *F. graminearum* sensu stricto Carm3 or *F. cerealis* Carm1 inoculated individually. Pots spiked with uninoculated cornmeal (20 g/kg of soil) were used as the control. Soybean plants were grown in a greenhouse at approximately 20 ± 3°C with 16 h of supplemental light until the V3 growth stage and evaluated for root rot symptoms (nine plants for each treatment) on a 0-to-4 scale, as described by Esmaeili Taheri et al. (2011).

Surface-sterilized wheat grains and soybean roots from each treatment (three plants in each treatment) were plot dried, plated on potato

Table 2. List of primers used during the current study^a

Primer	Sequence (5' to 3')	Direction	Tm (°C)	Target	Reference
ef1	ATGGGTAAGGARGACAAGAC	Forward	52.7	<i>TEF1α</i>	O'Donnell et al. 1998
ef2	GGARGTACCAGTSATCATGTT	Reverse	53.1	<i>TEF1α</i>	O'Donnell et al. 1998
Fg16F	CTCCGGATATGTTGCGTCAA	Forward	55	<i>Fg16</i>	Nicholson et al. 1998
Fg16R	GGTAGGTATCCGACATGGCAA	Reverse	56.6	<i>Fg16</i>	Nicholson et al. 1998
UBC85-F	GCAGGGTTTGAATCCGAGAC	Forward	56.1	<i>SCAR85</i>	Schilling et al. 1996
UBC85-R	AGAAATGGAGCTACCAACGGC	Reverse	57.4	<i>SCAR85</i>	Schilling et al. 1996
12CON	CATGAGCATGGTGATGTC	Reverse	50.8	<i>Tri12</i>	Ward et al. 2008
12-3F	CTTTGGCAAGCCCGTGCA	Forward	59.5	<i>Tri12</i>	Ward et al. 2008
12-15F	TACAGCGGTGCGCAACTTC	Forward	55.3	<i>Tri12</i>	Ward et al. 2008
12NF	TCTCCTCGTTGTATCTGG	Forward	50.5	<i>Tri12</i>	Ward et al. 2008
FusF	TGGGTAARGAGGASAAGACTACC	Forward	58.8	<i>TEF1α</i>	This study
mh1	GGTCACTTGATCTACCACTG	Forward	52.4	<i>TEF1α</i>	This study
mh2	GACAACATACCAATGACGGT	Reverse	52.7	<i>TEF1α</i>	This study
FgssF	CTTTGTCGTAATTTTTCYCCC	Forward	49.1	<i>TEF1α</i>	This study
FgssR	TATGAGCCCCACCGGG	Reverse	57.1	<i>TEF1α</i>	This study

^a Melting temperature (Tm), target gene or region (Target), and reference for each primer is indicated. *TEF1α* = translational elongation factor 1α and *SCAR* = sequence-characterized amplified region.

dextrose agar (PDA) medium, and incubated at 22 to 24°C for 7 days. Emerging *Fusarium* colonies from each treatment were purified and DNA was extracted from three *Fusarium* colonies in each treatment using the GenElute plant gDNA miniprep kit (Sigma-Aldrich, Canada) based on the manufacturer's recommendations. The *TEF1* α gene was amplified by PCR (as described above) and sequenced for species identification. The extracted DNA (three replicates in each treatment) was used as a template in the PCR-RFLP protocol as described above to detect *F. graminearum* sensu stricto and *F. cerealis* within the infected wheat and soybean tissues and to differentiate *F. graminearum* sensu stricto from other species, especially the closely related *F. cerealis* pathogen.

Results

Fg16F/Fg16R and UBC85F/UBC85R amplification. The Fg16F/R primer pair is used to detect different *Fusarium* spp. within the FGSC. This primer pair produces PCR fragments with variable length depending on the species: *F. brasiliicum* and *F. meridionale* produce a 500-bp fragment, while *F. nepalense*, *F. gerlachii*, and *F. aethiopicum* produce 400-bp fragments and *F. vorosii* produces a 390-bp fragment. All *F. graminearum* sensu stricto isolates investigated during the current study produce a 400-bp fragment when amplified with Fg16F and Fg16R primers. Some species within the FGSC did not produce any PCR amplicons (Fig. 2A, lanes 11, 17,

and 24), and all other species outside the FGSC (Fig. 2A, lanes 1 to 10) gave negative results and did not produce any amplicons. Similarly, SCAR85 was amplified selectively from *Fusarium* spp. within the FGSC using UBC85F and UBC85R primers. The UBC85F/R primer pair produce a 332-bp PCR amplicon in some species of the FGSC (Fig. 2B, lanes 11 to 13, 17, 19 to 22, and 24), whereas some others within the FGSC (Fig. 2B, lanes 14 to 16, 18, and 23) and other species not belonging to FGSC (Fig. 2B, lanes 1 to 10) showed no bands. Both Fg16F/R and UBC85F/R primer pairs can detect and differentiate species belonging to FGSC from any other *Fusarium* sp. but these primer pairs are not suitable to accurately differentiate between all species within the FGSC, and many false negatives can also be obtained. These two primer pairs were also not suitable for specific detection of *F. graminearum* sensu stricto.

PCR-RFLP for specific detection of *F. graminearum* sensu stricto. The *Hpa*II restriction enzyme was chosen to develop a PCR-RFLP marker specific for *F. graminearum* sensu stricto because it provided a species-specific restriction pattern that can differentiate *F. graminearum* sensu stricto among all other *Fusarium* spp. Based on the alignment for the *TEF1* α sequences (approximately 700 bp; amplified with the ef1/ef2 primer pair), the restriction site for *Hpa*II (5'-CCGG-3') was found in all investigated *Fusarium* spp. at two positions and found in *F. graminearum* sensu stricto at three positions (Fig. 1). Restriction digestion of the ef1/ef2 fragments

Fig. 2. Specific molecular detection of *Fusarium graminearum* sensu stricto. **A**, The commonly used Fg16F/R primer pair detects species within the *F. graminearum* species complex (FGSC); however, the size of the generated PCR band is not uniform. Bands ranged from 350 to 500 bp based on the species. **B**, Amplification of the sequence-characterized amplified region 85 region with the UBC85 primers, a 332-bp band obtained in the FGSC. **C**, PCR restriction fragment length polymorphism analysis of the translational elongation factor 1 α gene that was amplified with the primer pair mh1/mh2; the generated 459-bp amplicons were subjected to *Hpa*II-restriction digestion, and only *F. graminearum* sensu stricto (lanes 12 to 16) PCR amplicons produced a specific pattern consisting of two bands (199 and 260 bp). All other *Fusarium* spp., including the FGSC, did not produce this specific pattern and a single 450-bp band was observed. Using FG16F/R and UBC85 primers did not specifically differentiate *F. graminearum* sensu stricto from other species of the FGSC in addition to the presence of several false negatives within the FGSC group. Lane 1, *F. oxysporum*; lane 2, *F. redolens*; lane 3, *F. avenaceum*; lane 4, *F. acuminatum*; lane 5, *F. equiseti*; lane 6, *F. incarnatum*; lane 7, *F. poae*; lane 8, *F. culmorum*; lanes 9 and 10, *F. cerealis*; lane 11, *F. mesoamericanum*; lanes 12 to 16, *F. graminearum* sensu stricto; lane 17, *F. boothii*; lane 18, *F. brasiliicum*; lane 19, *F. meridionale*; lane 20, *F. nepalense*; lane 21, *F. vorosii*; lane 22, *F. gerlachii*; lane 23, *F. aethiopicum*; and lane 24, *F. acacia-mearnsii*.

produced a unique pattern to all *F. graminearum* sensu stricto isolates consisting of four fragments of 20 + 180 + 200 + 300 bp, while other *Fusarium* spp. produce a different restriction pattern consisting of three restriction fragments: 20 + 180 + 500 bp (Supplementary Fig. S1). For better visualization, a new primer pair, mh1/mh2, that binds conserved sequences in all *Fusarium* spp. was designed to amplify a portion of the *TEF1α* gene with one *HpaII* restriction site in *F. graminearum* sensu stricto only. The mh1 and mh2 primers amplify a 459-bp PCR fragment from all *Fusarium* spp., and the restriction digestion of the mh1/mh2 fragment produces a pattern unique to all *F. graminearum* sensu stricto isolates (two fragments: 199 bp + 260 bp), while any other *Fusarium* spp. were not affected by the *HpaII* enzyme and gave a 459-bp fragment (Fig. 2C). The newly designed PCR-RFLP marker accurately and selectively distinguishes *F. graminearum* sensu stricto from all other *Fusarium* spp., including members of FGSC. The capability of *HpaII* enzyme to digest its DNA target site in a short time has been investigated and results showed that 15 U of *HpaII* can efficiently and rapidly digest approximately 400 ng of mh1/mh2 PCR amplicons in 10 min (Supplementary Fig. S2). The FastDigest *HpaII* enzyme (15 U) can also completely digest approximately 400 ng of mh1/mh2 PCR amplicons in 5 min (Fig. 2C).

Specificity and sensitivity of the mh1/mh2 PCR primer pair. Specificity of the mh1/mh2 primer pair was tested with several *Fusarium* spp. and all isolates showed strong PCR amplification and yielded a single band, with a 459-bp fragment from all tested *Fusarium* spp. No amplification occurred when the mh1/mh2 primer pair was tested with gDNA from other fungal pathogens involved in FHB and FRR diseases in cereals and soybean, respectively, such as *C. spathianum* and *R. solani*. Other fungal pathogens such as *S. sclerotiorum* and *D. caulivora* produced 500- and 550-bp bands, respectively, but *HpaII* did not cleave these bands. Sensitivity of the mh1/mh2 primer pair was tested with different concentrations of pure *F. graminearum* sensu stricto gDNA templates (from 100 ng down to 1 fg). The mh1/mh2 primer pair, when used at 200 μM, can detect *F. graminearum* sensu stricto gDNA templates down to 10 pg.

In silico restriction digestion of the *TEF1α* gene. During the current study, the newly designed PCR-RFLP marker was tested in vitro with 19 *Fusarium* spp. (Fig. 2). The in vitro results showed that the *HpaII* PCR-RFLP marker can efficiently detect *F. graminearum* sensu stricto species and differentiate this pathogen among closely related *Fusarium* spp. within the FGSC and other related *Fusarium* pathogens involved in soybean root rot and FHB (Table 1). To confirm the accuracy of the *HpaII* PCR-RFLP marker and its unique specificity to *F. graminearum* sensu stricto, more *TEF1α* sequences have been retrieved from GenBank to be analyzed for the presence or absence of the *HpaII* restriction site. *TEF1α* sequences of species in Table 1 have been used as query to retrieve similar sequences to each species from different plant hosts in different geographic regions (Supplementary Table S1). In total, 85 *Fusarium TEF1α* sequences were analyzed in silico with the RestrictionMapper program (version 3), and results confirmed that only mh1/mh2 PCR fragments from *F. graminearum* sensu stricto isolates (isolated from different hosts in different geographic regions) were digested with *HpaII* enzyme and produced the characteristic restriction pattern as obtained during the in vitro experiments (199 + 260 bp). On the other hand, all other *Fusarium* spp. were not digested and a 450-bp PCR band was obtained. A phylogenetic tree was generated based on the *TEF1α* sequences of different *Fusarium* spp. involved in FHB and FRR (Fig. 3). DNA sequence logos for the *HpaII* restriction site and flanking sequences (10 upstream and 10 downstream nucleotides) clearly showed that the *HpaII* restriction site is unique to all *F. graminearum* sensu stricto isolates and absent in all other *Fusarium* spp.

Detection of *F. graminearum* sensu stricto in infected plant tissues using the *HpaII* PCR-RFLP tool. Both *F. cerealis* (Carm1) and *F. graminearum* sensu stricto (Carm3) were able to infect wheat and soybean and produce FHB and FRR symptoms in wheat and soybean, respectively. The presence of both *F. graminearum* sensu stricto and *F. cerealis* in infected plant tissues was confirmed by re-isolation of the pathogen on PDA medium. The pathogens were

reisolated from a subset of infected plants and identified morphologically as *F. graminearum* sensu stricto and *F. cerealis*. DNA was extracted from the reisolated *Fusarium* pathogens and *TEF1α* sequences were found to be 100% identical to *F. graminearum* sensu stricto and *F. cerealis*. FRR symptoms in soybean root at V1 and V3 growth stages are shown in Figure 4A, and FHB symptoms at wheat at full maturation stage are shown in Figure 4B (see Supplementary Table S2 for FRR and FHB scores). The mh1/mh2 primer pair was used to detect *F. graminearum* sensu stricto and *F. cerealis* in infected soybean and wheat plants. A 459-bp PCR amplicon was obtained using the mh1/mh2 primer pair from soybean roots infected with *F. graminearum* sensu stricto at V1 and V3 growth stages, as

Fig. 3. Phylogenetic tree based on the translational elongation factor 1α (*TEF1α*) sequences of different *Fusarium* spp. involved in Fusarium head blight and Fusarium root rot diseases in wheat and soybean, respectively. The tree topology based on neighbor-joining analysis and the largest and smallest circles on nodes represent bootstrap support values (100 to 1,000 respectively). Numbers in the tree represent the GenBank accession number of the *TEF1α* nucleotide sequences used in the phylogenetic analysis. The tree is rooted with *Fusarium pseudograminearum* (KM434221). FGSC = *F. graminearum* species complex. Nucleotide sequence logos are shown for the *HpaII* restriction site and flanking sequences (10 upstream and 10 downstream nucleotides). The *HpaII* restriction site (5'-CCGG-3') is boxed in gray-line box. Logos were generated by the online program WebLogo, version 2.8.2 (Crooks et al. 2004) (<http://weblogo.berkeley.edu/logo.cgi>). See Supplementary Table S1 for detailed information about the host plant and geographic region.

well as from wheat spikes infected with the same pathogen. The same band was also obtained from soybean and wheat spikes infected with *F. cerealis* (Fig. 4C). The 459-bp bands from infected soybean and wheat tissues, when digested with *HpaII*, produced a single 459-bp band for *F. cerealis*. *HpaII* digested the *F. graminearum* sensu stricto band into two bands of 199 and 260 bp. The *HpaII* PCR-RFLP marker showed highly accurate detection of *F. graminearum* sensu stricto and was able to differentiate this pathogen from other closely related species.

***F. graminearum* sensu stricto specific primers.** As mentioned above, the Fg16F/R and the UBC58F/R primer pairs have been used to specifically detect members within the FGSC. During the current study, we attempted to validate these two commonly used primer pairs on a subset of *Fusarium* spp. but the results showed that neither primer pair could distinguish *F. graminearum* sensu stricto from other species in the FGSC (Fig. 2A and B). In addition, the two primer pairs can generate false negatives with several species and isolates within the FGSC, giving the possibility for misdetection of any of these *Fusarium* pathogens. During this study, sequence analysis of the *TEF1 α* gene in FGSC and other *Fusarium* spp. involved in FHB and FRR diseases to cereal and soybean crops, respectively, allowed for the design of two primers specific to *F. graminearum* sensu stricto (FgssF and FgssR). The unique *HpaII* restriction site was used to design these two primers, in which the 3' end of each primer was designed to pair with the 5'-CC-3' nucleotides in the enzyme's

restriction site (5'-CCGG-3'). Sequence similarities within the *TEF1 α* gene within the genus *Fusarium* are extremely high, and the most effective way to design species-specific primers is to make the few nucleotides in the 3' end of the primer specific to the species of interest. For successful DNA polymerase extension, the primer's 3' end should be an exact match to the DNA template. Another important factor for DNA polymerase extension is to design the primer with G or C at the 3' end for stronger binding. These two factors were considered when designing the FgssF and FgssR primers with the 5'-CC-3' 3' end that is unique and specific to *F. graminearum* sensu stricto. The FgssF primer was paired with ef2 while the FgssR primer was paired with FusF to amplify 354- and 368-bp fragments, respectively, with all isolates of the *F. graminearum* sensu stricto, whereas no bands were generated with all other *Fusarium* spp. belonging to the FGSC or any other *Fusarium* sp.

Both primer pairs (FgssF/ef2 and FusF/FgssR) were found to have strong single bands and have high sensitivity and specificity to *F. graminearum* sensu stricto, and no bands were generated with any other *Fusarium* spp. belonging to the FGSC or other *Fusarium* spp. involved in FHB and FRR diseases in cereal and soybean crops, respectively (Fig. 5). Sensitivity of both FusF/FgssR and FgssF/ef2 primer pairs was tested with different concentrations of pure *F. graminearum* sensu stricto gDNA templates (from 10 ng down to 100 fg). The FusF/FgssR primer pair, when used at 200 μ M, can detect *F. graminearum* sensu stricto gDNA templates down to 100 fg, while sensitivity of FgssF/ef2 primer pair was 1 pg (Fig. 5). We recommend using FusF/FgssR for specific detection of *F. graminearum* sensu stricto due to the higher sensitivity of this primer pair.

Discussion

Several approaches have been used for fungal classification and to define species limits of the genus *Fusarium*, in which classification based on morphology (i.e., known as "morphological species complex") was considered the main approach used to classify *Fusarium* spp. and was used by fungal taxonomists for over 200 years (Aoki et al. 2014; Cai et al. 2011; Nelson 1991; Summerell et al. 2010). Another concept is the "biological species concept", which describes species as groups of populations that interbreed with each other (Mayr 1940, 1963). There are limitations for both morphological and biological species concepts; in the former, there are not enough unique morphological characters for all species needed to be discriminated whereas, in the later, there are many *Fusarium* isolates that reproduce asexually and rarely produce sexual stages (Nelson 1991; Summerell et al. 2010). Recently, the "phylogenetic species concept", which defined species as the small monophyletic clade of organisms that share a derived character set (Cracraft 1983; Taylor et al. 2000), was developed and used to define species limits. This concept separates species based on converting differences in DNA sequence of selected genes into quantitative measurements of genetic relatedness (Summerell et al. 2010). Approximately 300 phylogenetic species were identified within the genus *Fusarium* based on molecular phylogenetics (i.e., phylogenetic species concept). Using the phylogenetic species recognition criterion, O'Donnell et al. (2000) divided *F. graminearum* into seven phylogenetically distinct species based on phylogenetic analysis of six single-copy genes. Other studies based on high-throughput multilocus genotyping with DNA sequences from 13 nuclear genes divide *F. graminearum* into 16 monophyletic species. These species are collectively referred to as the FGSC (Cai et al. 2011; O'Donnell et al. 2004; Sarver et al. 2011; Starkey et al. 2007). *F. graminearum* sensu stricto is considered the main FHB causative agent within the FGSC (*F. graminearum* sensu lato), and recently was found to show cross-pathogenicity and cause root rot of soybean. For this reason, specific detection of *F. graminearum* sensu stricto and differentiating this important toxigenic pathogen from other species in the FGSC and other closely related *Fusarium* spp. is considered an important issue for accurate disease diagnosis, better control, and minimizing the negative effects of mycotoxins. In the present study, we have developed two-step and one-step molecular tools to detect *F. graminearum* sensu stricto in vitro and in planta (i.e., infected wheat and soybean tissues) and

Fig. 4. Detection of *Fusarium graminearum* sensu stricto from infected soybean plants (with root rot symptoms) and infected wheat plants (with Fusarium head blight symptoms). **A.** Soybean plants infected with *F. cerealis* (*F. cer.*) and *F. graminearum* sensu stricto (*F. gra.*) in V1 and V3 growth stages. Noninfected soybean plants were used as control (Cont.). **B.** Wheat spikes infected with *F. cerealis* and *F. graminearum* sensu stricto; noninfected wheat spikes were used as control. **C.** Detection of *F. cerealis* and *F. graminearum* sensu stricto in both soybean roots and wheat spikes using the mh1/mh2 primer pair that produced 459-bp bands in both pathogens and no bands in the control. Only *F. graminearum* sensu stricto bands were recognized by the *HpaII* enzyme and produced two bands (199 and 260 bp).

differentiate this pathogen from other *Fusarium* pathogens involved in cereals and soybean diseases.

PCR-RFLP markers for *Fusarium* spp. PCR-RFLP markers based on ITS sequence variations have been developed to identify and study the genetic relationships between different *Fusarium* spp. (Cai et al. 2011; O'Donnell et al. 2004; Sarver et al. 2011; Starkey et al. 2007). The nuclear ribosomal ITS region has been used intensively as a marker for species identification since the development of the first universal primers to amplify this DNA region in fungi. Schoch et al. (2012) proposed using ITS as the sole universal barcode for fungi; however, the authors stated that this proposal will satisfy most but not all mycologists. Phylogenetically, closely related species (i.e., cryptic species such as the FGSC) differ by as little as as one to only a few nucleotide positions at the ITS level (Cai et al. 2011; O'Donnell et al. 2004; Sarver et al. 2011; Starkey et al. 2007). For this reason, Kiss (2012) did not consider ITS to be a sufficiently powerful tool to discriminate between closely related species.

The β -TUB which encodes for the structural proteins of microtubules and other structural components in eukaryotes was also used to differentiate between different plant and human pathogenic *Fusarium* spp. (O'Donnell et al. 2009; Yli-Mattila et al. 2004). Nosratabadi et al. (2018) evaluated the efficiency of PCR-RFLP based on the β -TUB region sequence to study genetic relationships in a total of 107 *Fusarium* isolates. The proposed PCR-RFLP protocol is based on amplifying the β -TUB gene and digesting the amplified fragments with three restriction enzymes, which allowed differentiation between *F. graminearum*, *F. culmorum*, and *F. cerealis* but not between other investigated *Fusarium* spp. For this reason, Nosratabadi et al. (2018) recommend that other genes must be evaluated to overcome the limitations of the β -TUB gene in differentiating these species.

The *TEF1 α* gene is considered the most frequently used sequence to define species boundaries within the genus *Fusarium* and has high phylogenetic utility because it is highly conserved and highly informative at the species level in *Fusarium*. In addition, no nonorthologous copies of this gene have been detected in the genus *Fusarium*. The *Fusarium* DNA sequence database (FUSARIUM-ID, v. 1.0), which is currently considered to be the most important tool for *Fusarium* sp. identification, is also based on *TEF1 α* sequences (Geiser et al. 2004). The *TEF1 α* gene was first used as a phylogenetic marker in fungi by O'Donnell et al. (1998) to study the evolutionary origins of *F. oxysporum* f. sp. *cubense*, the causal pathogen of Panama disease of banana. They also developed degenerative primers (ef1 and ef2) based on conserved nucleotide sequences within the *TEF1 α* gene shared by two ascomycetous fungi in the orders Hypocreales and Eurotiales, which can be used to partially amplify an approximately 700-bp fragment from the *TEF1 α* gene in a wide variety of ascomycetous fungi. The *TEF1 α* gene was used as a marker gene to study phylogenetic relationships within the genus *Fusarium* (Knutsen et al. 2004; Mbofung et al. 2007; O'Donnell et al. 2008) and also to detect and differentiate between different *Fusarium* spp. (Carbone and Kohn 1999; Garmendia et al. 2018; Zitnick-Anderson et al. 2018). More interestingly, Kristensen et al. (2005), studied the relationship between *Fusarium* phylogeny and toxin production potential and found a correlation between major phylogenetic lineages and the ability to synthesize particular classes of toxins. Specific detection of *F. graminearum* sensu stricto is an extremely important issue due to the wide range of mycotoxins produced by isolates of this pathogen. Type-B trichothecenes are considered to be the most common mycotoxins produced by *F. graminearum* sensu stricto (DON, 15ADON, and 3ADON). The trichothecene chemotype produced by different species within the genus *Fusarium* seems to be dynamic and the pathogen can change this

Fig. 5. Specific detection of *Fusarium graminearum* sensu stricto with FusF/FgssR and FgssF/ef2 primer pairs. The newly designed FgssR and FgssF primers were combined with FusF and ef2 primers to produce 368- or 354-bp fragments, respectively. Only *F. graminearum* sensu stricto (Fgss) isolates gave positive amplification whereas other species within the *F. graminearum* species complex (FGSC) or other closely related *Fusarium* spp. yielded no bands. Sensitivity of both primer pairs to different concentrations of pure *F. graminearum* sensu stricto (Fgss) genomic DNA (gDNA) templates (from 10 ng down to 100 fg) is indicated. Both FusF/FgssR and FgssF/ef2 primer pairs also gave negative results with other fungal pathogens such as *Rhizoctonia solani*, *Phytophthora infestans*, *Sclerotinia sclerotiorum*, and *Diaporthe caulivora* (data not shown). No bands were obtained when gDNA from soybean and wheat was used as template.

chemotype in response to surrounding biotic and abiotic factors. Recently, an *F. graminearum* population with 3ADON was found to replace the predominant 15DON population in Canada (Ward et al. 2008). More interestingly, a population of *F. graminearum* isolated from wheat in North America, which produced none of the known trichothecene mycotoxins despite causing normal FHB disease symptoms, has been identified. A new trichothecene chemotype (named NX-2) was characterized by liquid-chromatography tandem mass spectrometry. This new trichothecene mycotoxin is of type A due to the absence of the keto group at C-8 (Varga et al. 2015). This capability of *F. graminearum* sensu stricto to produce several toxins and even to switch from one chemotype to another obviously calls for an accurate specific detection. Species of the FGSC are undistinguishable morphologically and, due to their high similarity at the molecular level, there has been no specific molecular marker for *F. graminearum* sensu stricto. Therefore, the PCR-RFLP tool and the species-specific PCR primers developed in the current study will be highly valuable in this regard.

Several *TEF1 α* -based PCR-RFLP tools have been developed to differentiate between different *Fusarium* spp. Nitschke et al. (2009) have conducted in silico analysis of *TEF1 α* sequences from 65 *Fusarium* isolates belonging to 18 species isolated from sugar beet. They developed a reliable PCR-RFLP tool to differentiate 17 of 18 species using two subsequent restriction digests of *TEF1 α* PCR-amplified fragments. The study included *F. graminearum* and other *Fusarium* spp. involved in sugar beet diseases as well as other *Fusarium* spp. involved in other crops commonly grown with sugar beet in rotations such as wheat and corn; however, this study did not include any other FGSC representatives. Recently, a PCR-RFLP tool based on the *TEF1 α* sequence was developed to detect and differentiate *F. graminearum* sensu stricto from the remaining species within the FGSC and from other *Fusarium* spp. causing FHB in cereals (Garmendia et al. 2018). This PCR-RFLP tool was designed to amplify the *TEF1 α* gene with the commonly used ef1/2 primer pair and digest the amplified PCR fragments with *Bsa*HI enzyme. The enzyme's restriction site (5'-GRCGYC-3', where, R could be A or G and Y could be C or T) is only found in *F. graminearum* sensu stricto and is absent in other FGSC species and other closely related *Fusarium* spp. A unique pattern was only obtained with *F. graminearum* sensu stricto (two bands: 367 and 291 bp), whereas other *Fusarium* spp. were not cleaved. The multiple recognition sites for *Bsa*HI (5'-GRCGYC-3'; GACGCC, GACGTC, GGCGCC, and GGCGTC) affect its specificity to *F. graminearum* sensu stricto, and other *Fusarium* spp. could be recognized and cleaved by the *Bsa*HI enzyme. We have analyzed the *TEF1 α* sequences from the FGSC and other closely related *Fusarium* spp. in silico, and results showed that several *Fusarium* spp. other than *F. graminearum* sensu stricto contain the restriction site of the *Bsa*HI enzyme (5'-GRCGYC-3'). *F. asiaticum* (KY466738), *F. culmorum* (KY659120), and *F. pseudograminearum* (EF428635 and EF428644) have the exact restriction site of *Bsa*HI (5'-GGCGTC-3') at the same position and will give the same pattern as *F. graminearum* sensu stricto if tested with the *Bsa*HI PCR-RFLP protocol. *F. chlamydosporum* (KY211036 and KF918546) also has a *Bsa*HI restriction site (5'-GGCGCC-3') and will also give the same pattern as *F. graminearum* sensu stricto if tested with the *Bsa*HI PCR-RFLP marker.

In the current study, the *TEF1 α* gene was amplified with the newly designed primers mh1 and mh2 to produce a 459-bp PCR amplicon. Subsequent RFLP to the PCR amplicons with *Hpa*II restriction enzyme showed that only *F. graminearum* sensu stricto isolates were cleaved by *Hpa*II to give a unique pattern specific for this important pathogen, consisting of two fragments (199 and 460 bp), whereas all other *Fusarium* spp. in the FGSC and other *Fusarium* spp. involved in FHB and FRR diseases in cereals and soybean, respectively, were not cleaved by the enzyme and a single band (459 bp) was obtained by these species. Additional in silico analysis for 84 *TEF1 α* gene sequences of *F. graminearum* sensu stricto and other species of the FGSC as well as other closely related *Fusarium* spp. isolated from different hosts in different places all over the world was conducted. The aim of this sequence analysis was to confirm that the *Hpa*II

restriction site is only found in *F. graminearum* sensu stricto and is absent in any other *Fusarium* sp. regardless of the geographic region and host. The results confirmed that the *Hpa*II restriction site is unique to *F. graminearum* sensu stricto and can be considered a species-specific signature sequence that makes *Hpa*II PCR-RFLP to *TEF1 α* PCR amplicons a suitable fast and accurate molecular tool to detect and differentiate *F. graminearum* sensu stricto from any other *Fusarium* spp.

***F. graminearum* sensu stricto-specific primers.** Several molecular tools have been developed to detect and differentiate between species within the FGSC. Among these molecular tools, the Fg16F/R primer pair has been used extensively to detect FGSC members and differentiate between these closely related species by producing polymorphic PCR products (400 to 580 bp). The Fg16F and Fg16R primers have been used to study genetic diversity among different FGSC isolates, to differentiate between isolates within this species complex, and also to discriminate between the FGSC and other closely related *Fusarium* spp. Nicholson and coworkers (1998) initially used Fg16F and Fg16R primers to differentiate between FGSC members and other closely related *Fusarium* spp. such as *F. culmorum* and *F. crookwellense* (syn. *F. cerealis*). PCR amplification using these primers produced polymorphic products (400 to 500 bp) with the FGSC but no products with DNA from other *Fusarium* spp. Carter et al. (2002) used the Fg16F/R primer pair to study the genetic diversity among FGSC isolates and six SCAR groups have been obtained, each with a different PCR product size (420, 510, 540, 580, 520, and 400 bp for SCAR types 1 to 6, respectively). Qu et al. (2008) have also used Fg16 F/R-based SCAR analysis to detect and differentiate between *F. graminearum* sensu stricto and *F. asiaticum* isolates. Two PCR product sizes were observed, corresponding to SCAR type 1 (420 bp: *F. graminearum*) and SCAR type 5 (520 bp: *F. asiaticum*) reported by Carter et al. (2002). The Fg16F and Fg16R primers are considered an important tool to detect FGSC isolates and differentiate between these important pathogens and other closely related *Fusarium* spp.; however, the Fg16F/R primer pair cannot specifically detect and differentiate between *F. graminearum* sensu stricto (the major causal agent of FHB of wheat worldwide) and other *Fusarium* spp. in the FGSC such as *F. nepalense*, *F. gerlachii*, and *F. aethiopicum*, because these species produce amplicons (420 bp) identical to those of *F. graminearum* sensu stricto when amplified with Fg16F/R. Other *Fusarium* spp. within the FGSC produce amplicons with Fg16F/R that are very close in size (400 to 500 bp), which makes differentiation between species in this complex difficult. A recent study using representatives of all described FGSC species showed that, using the Fg16F/R primer set to distinguish between some species in the FGSC, including *F. graminearum* sensu stricto, is difficult or impossible due to the narrow range of amplicon sizes obtained with this primer pair (Garmendia et al. 2018). The UB85F/R primer set is specific for FGSC and produces monomorphic amplicons (332 bp) (Schilling et al. 1996) with all *F. graminearum* sensu lato species. For this reason, this primer pair is not suitable to differentiate between species in the FGSC and also for specific detection of *F. graminearum* sensu stricto.

In the present work, specificity of the newly designed primer FgssR has been tested and results showed that this primer, when combined with the FusF primer, can only detect isolates of the *F. graminearum* sensu stricto and produce 368-bp amplicons, while all other *Fusarium* spp. in the FGSC produced no amplicons. The specificity of the FusF/FgssR primer pair was also tested with other *Fusarium* spp. involved in FHB and FRR in cereals and soybean, respectively, and results confirms the specificity of FusF/FgssR to *F. graminearum* sensu stricto. The *Hpa*II PCR-RFLP and the FgssR-specific primer presented during the current work would effectively and accurately detect and differentiate *F. graminearum* sensu stricto from all other *Fusarium* spp., and can reduce time, effort, and cost in detecting this pathogen.

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