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TRI12 based quantitative real-time PCR assays reveal the distribution of trichothecene genotypes of *F. graminearum* and *F. culmorum* isolates in Danish small grain cereals

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ABSTRACT

Quantitative real-time PCR assays, based on polymorphisms in the *TRI12* gene of the trichothecene pathway, were developed to identify and quantify the trichothecene genotypes producing 3-acetyl-deoxynivalenol (3ADON), 15-acetyl-deoxynivalenol (15ADON) or nivalenol (NIV) in the *Fusarium graminearum* species complex, *Fusarium culmorum*, *Fusarium cerealis* and *Fusarium pseudograminearum*. These assays were applied on a total of 378 field samples of cereal grain of wheat, barley, triticale, rye and oats collected from 2003 to 2007 to study the trichothecene genotype composition in Danish cereals. The three genotypes, 3ADON, 15ADON and NIV were found in all five cereal species, great annual variation in the occurrence of the trichothecene genotypes was evident with considerable variation between the samples. 3ADON was the dominant genotype in barley, triticale, rye and oats while 15ADON was most dominant in wheat. The NIV genotype was found at low levels in most samples. Study of genotype composition within the Danish *F. graminearum* and *F. culmorum* population was based on principal component analysis (PCA). PCA revealed that the dominating genotype of *F. graminearum* in wheat is 15ADON. For barley, the PCA analysis indicated that the *F. graminearum* population consisted of all three genotypes, and in triticale, the *F. graminearum* population consisted mainly of 15ADON genotype. *F. culmorum*/*F. cerealis* showed correlation to the NIV genotype in wheat and triticale but not in barley. *F. culmorum*/*F. cerealis* also showed some correlation to 3ADON especially in wheat and triticale. Selected wheat and barley samples from 1957 to 2000 showed low amounts of *F. graminearum* and *F. culmorum* in general but with a dominance of the 3ADON genotype. 15ADON was not detected in these samples, except for very low amounts in the sample representing the years from 1997 to 2000. Detection of low amounts of the 15ADON genotype in these historical samples and the relatively high amounts of 15ADON genotype in 2003 and following years correspond well with the occurrence of *F. graminearum* and indicates that the 15ADON genotype was introduced along with *F. graminearum* around 2000. The amounts of the 3ADON and 15ADON genotypes correlated well with the total amount of DON whereas the amounts of NIV genotype correlated well with the amount of NIV in wheat and triticale but not in barley where the results indicate that *Fusarium poae* may also contribute to the NIV content.

1. Introduction

Fusarium head blight (FHB) is an important disease in small grain cereals worldwide, caused by a complex of *Fusarium* species along with *Microdochium nivale* and *Microdochium majus*. *Fusarium graminearum* is the species most commonly associated with FHB worldwide and belongs to the *F. graminearum* species complex, which consists of at least 13 phylogenetic distinct species (O'Donnell et al., 2000; O'Donnell et al., 2004; Starkey et al., 2007; Yli-Mattila et al., 2009). A recent extensive study of grains of wheat, barley, triticale, oats and rye sampled in Denmark from 2003 to 2007 using real-time PCR targeting the *F. graminearum* complex except *Fusarium brasilicum* and *Fusarium cortaderiae* has shown that the *F. graminearum* species complex occurs frequently and is dominant in Denmark, especially in wheat and triticale (Nielsen et al., 2011). The *F. graminearum* species complex in Denmark is most likely represented solely by *F. graminearum sensu stricto* designated lineage 7 which has been found to be the dominant or sole lineage in European countries (Láday et al., 2004; O'Donnell et al., 2000; O'Donnell et al., 2004; Tóth et al., 2005). A shift in the *Fusarium* population with an increase in *F. graminearum* has been reported in Holland (Waalwijk et al., 2003), Poland (Stepien et al., 2008) and Denmark (Nielsen et al., 2011). This change is regarded as a consequence of changed agricultural practices with increased maize cropping as one of the main factors (Champeil et al., 2004; Parry et al., 1995). *F. graminearum* and *Fusarium culmorum* both produce mycotoxins which are of great concern, as they

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are toxic to humans and animals. The principal toxins produced by the *F. graminearum* species complex and other closely related species, including *F. culmorum*, *Fusarium cerealis* and *Fusarium pseudograminearum*, are the type B trichothecenes; deoxynivalenol (DON) and nivalenol (NIV) (Jennings et al., 2004a; O'Donnell et al., 2000; Ward et al., 2008). Throughout Europe (Bottalico and Perrone, 2002), as well as in Denmark, DON is the most frequently detected trichothecene toxin in wheat (Nielsen et al., 2011). Studies on the effect on cell metabolism and cell proliferation have indicated that NIV is more toxic to humans and animals than DON (Luongo et al., 2008; Minervini et al., 2004) whereas other studies of DNA-synthesis have shown no difference in toxicity (Eriksen et al., 2004). In 2006 The European Commission set legislative limits for *Fusarium* produced mycotoxins in cereal grain and cereal-based products intended for human consumption and animal consumption with limits of DON of 1250 ppb and 900 ppb respectively in unprocessed grain (Anon., 2006a; Anon., 2006b). Due to co-occurrence with DON no legislative limit has been set for NIV as the amount of NIV usually follows the levels of DON and the legislation for DON will protect humans from unacceptable exposure of this toxin (Leslie et al., 2008). Chemotype characterization of *F. graminearum* and *F. culmorum* has been used extensively to determine their potential for production of NIV, DON and its acetylated derivatives 3-acetyldeoxynivalenol (3ADON) or 15-acetyldeoxynivalenol (15ADON) (Desjardins, 1996). Chemotyping has also been applied to obtain further information on the population structure and changes over time and space of the trichothecene genotype distribution of *F. graminearum* and *F. culmorum* (Starkey et al., 2007; Ward et al., 2008). The 15ADON chemotype of *F. graminearum* has been identified previously as the predominant chemotype in the United States, with isolates of the NIV and 3ADON type detected less frequently (Gale et al., 2011; Gale et al., 2007; Starkey et al., 2007). In Canada and North Dakota, a major shift from 15ADON isolates of *F. graminearum* towards 3ADON isolates has taken place (Puri and Zhong, 2010; Ward et al., 2008). In the Russian Far East the number of 3ADON isolates of *F. graminearum* compared with 15ADON isolates has also been increasing (Yli-Mattila and Gagkaeva, 2010). 3ADON isolates have been shown to grow faster and produce more spores (Ward et al., 2008) and more DON *in vitro* as well as under field conditions (von der Ohe et al., 2010; Ward et al., 2008). In terms of differences in the severity of FHB disease caused by the two chemotypes, the results are inconclusive. Single-floret inoculation studies of different wheat genotypes have shown the 3ADON isolates to cause a higher disease severity than the 15ADON isolates in some varieties (Puri and Zhong, 2010) whereas no significant differences in disease severity could be found under field conditions for other varieties (von der Ohe et al., 2010).

A phylogenetic study of genes in the main trichothecene gene cluster showed that the B trichothecene producing species group according to chemotype rather than species, indicating that DNA polymorphisms within this gene cluster have persisted throughout the evolutionary history of this clade (Ward et al., 2002). Clustering according to chemotype was strongly supported in individual phylogenies of the *TRI3*, *TRI11* and *TRI12* genes, although variation within these genes is not entirely responsible for the observed differences in chemotype (Alexander et al., 2011). Based on these polymorphisms genotype specific PCR tests for distinguishing 3ADON, 15ADON and NIV producing isolates of the *F. graminearum* species complex, *F. culmorum*, *F. cerealis* and *F. pseudograminearum* have been developed (Kulik, 2011; Ward et al., 2002). Conventional PCR tests have been used to determine the trichothecene genotype of individual isolates in order to study the distribution of these in different geographical areas (Chandler et al., 2003; Pasquali et al., 2010; Quarta et al., 2006; Quarta et al., 2005; Reynoso et al., 2011; Sampietro et al., 2010; Wang et al., 2008; Ward et al., 2002; Yli-Mattila, 2010; Yli-Mattila and Gagkaeva, 2010). In the present study, we aimed at developing quantitative real-time PCR (QPCR) methods for quantification of the total biomass of the 3ADON, 15ADON and NIV trichothecene genotypes within the *F. graminearum* complex including all lineages described by O'Donnell et al. (2004) in addition to *F. culmorum*, *F. cerealis* and *F. pseudograminearum*. The three designed QPCR all target the *TRI12* gene and were applied in a study of the genotype composition of the *F. graminearum* and *F. culmorum*/*F. cerealis* population in Danish cereal small grain samples of wheat, barley, triticale, rye and oats collected from 2003 through 2007 as well as some few historical grain samples of wheat and barley sampled prior to 2000. Results of this study were used in the verification of the potential for such QPCR assays to be used in prediction of the mycotoxin content by correlating QPCR data with the actual mycotoxin content in the grain samples.

2. Materials and methods

2.1. Primers for quantitative real-time PCR

3ADON, 15ADON and NIV genotype specific primers were designed based on sequences of *TRI12* from *F. graminearum*, *Fusarium asiaticum*, *Fusarium austroamericanum*, *Fusarium boothii*, *F. cortaderiae*, *Fusarium meridionale*, *Fusarium mesoamericanum* and *Fusarium acaciae-mearnsii* in the *F. graminearum* species complex as well as *F. culmorum*, *F. cerealis* and *F. pseudograminearum*. Sequences were retrieved from GenBank and aligned using CLC Sequence Viewer. Primers were designed using a combination of the Primers express Program version 2.0 (Applied Biosystems, Foster City, CA, USA) and the alignments (Table 1). Test of the primers was carried out by comparing primer sequences to the nucleotide sequence database in GenBank using BLAST and by running PCR on selected isolates of *Fusarium* spp.

2.2. Fungal isolates and field samples

Fungal isolates of known trichothecene genotype were used for the specificity test, standard curves and positive controls (Table 2). Isolates were grown on potato dextrose agar (PDA) at 22 °C under 12 h light and 12 h darkness. Field samples of cereal grain were from 2003 to 2007 and consisted of harvested seeds from Danish fields (Nielsen et al., 2011). Milled grain samples of winter wheat and spring barley grown from 1957 to 2000 were obtained from the ASKOV long-term studies of animal manure and mineral fertilizers situated in Mid-Jutland (Christensen et al., 2006). Each sample was provided as a pooled sample consisting of four sub-samples each representing one year. Each of these subsamples was pooled from four to five replicates from each year. The pooled samples were stored as milled grain in closed containers at room temperature. In the present study a total of 227 wheat, 61 barley, 53 triticale, 17 rye and 20 oats grain samples along with 8 historical samples of wheat and barley, respectively, were studied. All samples have been previously analysed for species composition of the *Fusarium* head blight complex (Nielsen

Table 1
Specific primers used for real-time PCR amplifying the 3ADON, 15ADON and NIV trichothecene genotypes in the *TRI12* gene.

Target	Primer name	Sequence (5′-3′)	Product size (bp)	Position in *TRI12*
3ADON	3ADONf	AACATGATCGGTGAGGTATCGA	60	16605–16665[a]
	3ADONr	CCATGGCGCTGGGAGTT		
15ADON	15ADONfwd	GTTTCGATATTCATTGGAAAGCTAC	57	16451–16508[b]
	15ADONrev	CAAATAAGTATCGTCTGAAATTGGAAA		
NIV	NIVf	GCCCATATTCGCGACAATGT	77	18683–18760 [c]
	NIVr	GGCGAACTGATGAGTAACAAAACC		

[a] Position in the sequence of the 3ADON producing *F. graminearum* strain NRRL 28336, gene accession AY102584.
[b] Position in the sequence of the 15ADON producing *F. graminearum* strain NRRL 6394 gene accession AY102605.
[c] Position in the sequence of the NIV producing *F. graminearum* strain NRRL 28439 gene accession AY102587.