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Altered trichothecene biosynthesis in *TRI6*-silenced transformants of *Fusarium culmorum* influences the severity of crown and foot rot on durum wheat seedlings

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SUMMARY

An RNA silencing construct was used to alter mycotoxin production in the plant pathogenic fungus *Fusarium culmorum*, the incitant of crown and foot rot on wheat. The transformation of a wild-type strain and its nitrate reductase-deficient mutant with inverted repeat transgenes (IRTs) containing sequences corresponding to the trichothecene regulatory gene *TRI6* was achieved using hygromycin B resistance as a selectable marker. Southern analysis revealed a variety of integration patterns of the *TRI6* IRT. One transformant underwent homologous recombination with deletion of the endogenous *TRI6* gene, whereas, in another transformant, the *TRI6* IRT was not integrated into the genome. The *TRI6* IRT did not alter the physiological characteristics, such as spore production, pigmentation or growth rate, on solid media. In most transformants, a high *TRI6* amplification signal was detected by quantitative reverse transcription-polymerase chain reaction, corresponding to a *TRI6*-hybridizing smear of degraded fragments by Northern analysis, whereas *TRI5* expression decreased compared with the respective non-transformed strain. Four transformants showed increased *TRI5* expression, which was correlated with a dramatic (up to 28-fold) augmentation of deoxynivalenol production. Pathogenicity assays on durum wheat seedlings confirmed that impairment of deoxynivalenol production in the *TRI6* IRT transformants correlated with a loss of virulence, with decreased disease indices ranging from 40% to 80% in nine silenced strains, whereas the overproducing transformants displayed higher virulence compared with the wild-type.

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INTRODUCTION

Fusarium culmorum (W.G. Smith) Sacc. is a major fungal pathogen of wheat, causing two forms of disease, namely crown and foot rot (CFR) and fusarium head blight (FHB) (Corazza *et al*., 2002; Smiley and Patterson, 1996; Wagacha and Muthomi, 2007; Wiese, 1987). The severity of CFR incited by *F. culmorum* is usually greater in dry soils and is commonly associated with warm temperatures (Cariddi and Catalano, 1990). These conditions are dominant in central and southern Italy, where durum wheat is commonly grown. Disease symptoms may include seedling death before or soon after emergence, brown lesions on the stem base, tiller abortion and the formation of whiteheads, containing shrivelled grain or no grain at all. Consequently, significant yield losses are reported and the grain often becomes contaminated with mycotoxins.

Among the most bioactive compounds are trichothecenes, i.e. sesquiterpene epoxides, which are able to inhibit eukaryotic protein synthesis (Wei and McLaughlin, 1974) and may cause toxicoses in humans or animals consuming contaminated food or feed (Sudakin, 2003). Trichothecenes may also induce apoptosis (Desmond *et al*., 2008; Yang *et al*., 2000) and have been suggested to play an important role in the aggressiveness of phytopathogenic *Fusarium* species towards plant hosts (Bai *et al*., 2002; Desjardins *et al*., 1996, 2000; Harris *et al*., 1999; Jansen *et al*., 2005; Maier *et al*., 2006; McCormick, 2003; Proctor *et al*., 1995, 2002; Ward *et al*., 2008; Zhang *et al*., 2010).

Type B trichothecenes, produced mainly by *Gibberella zeae* (Schwein.) Petch (anamorph: *Fusarium graminearum* Schwabe) and *F. culmorum*, are predominant in Europe. They include deoxynivalenol (DON), also known as vomitoxin, with its derivatives monoacetyldeoxynivalenols (3-AcDON, 15-AcDON), nivalenol

(NIV) and its acetylated form 4-acetylnivalenol or fusarenone X (Bottalico and Perrone, 2002; Goswami and Kistler, 2004).

CFR control is often difficult and mainly relies on preventative measures, such as crop rotation, the use of tolerant cultivars, burial of infested debris, reduced nitrogen fertilization and seed dressing with fungicides. However, the risk of residues and the potential for increased levels of mycotoxins in the grain under certain circumstances (Gardiner *et al*., 2009) may limit their use.

The biosynthesis of trichothecenes starts with the formation of trichodiene via the cyclization of farnesyl pyrophosphate. Trichodiene then undergoes a series of oxygenation, cyclization, isomerization and esterification steps to yield bioactive trichothecenes, such as DON and acetylated DON (Desjardins *et al*., 1993). At least 12 genes that encode enzymes catalysing most of these biosynthesis steps have been characterized and found to be located within a single core cluster in *G. zeae* and in the closely related species *Fusarium sporotrichioides* Sherb. (for reviews, see Alexander *et al*., 2009; Proctor *et al*., 2002).

TRI5, the first trichothecene biosynthetic gene identified in *F. sporotrichioides*, encodes the enzyme trichodiene synthase (Hohn and Beremand, 1989). In *G. zeae*, *TRI5* function was determined by transformation-mediated gene disruption experiments, resulting in mutants that were unable to carry out the first biosynthetic step and were incapable of producing DON or any of the trichothecene biosynthetic intermediates (Desjardins *et al*., 1996; Harris *et al*., 1999; Jansen *et al*., 2005; Maier *et al*., 2006). These mutants were reduced in virulence when grown on wheat (Proctor *et al*., 1995).

TRI6 encodes a Cys_2-His_2-type positive transcription factor that binds to the promoter regions of genes located in the core *TRI* cluster, as well as to *TRI1*, *TRI16* and *TRI101*, which are located outside of the *TRI* cluster (Alexander *et al*., 2009). Thus, this gene plays a crucial role in regulating the expression of multiple biosynthetic genes, including *TRI5*, and can be regarded as a potential molecular target when developing resistant wheat lines or new molecules that could reduce trichothecene biosynthesis.

A possible approach to the downregulation of gene expression in fungi and other eukaryotic organisms in a sequence-specific manner utilizes RNA silencing (Catalanotto *et al*., 2000; Cogoni and Macino, 1997; Denli and Hannon, 2003; Pickford and Cogoni, 2003; Waterhouse *et al*., 2001). Specific inhibition of gene expression by RNA silencing has been demonstrated in several filamentous fungi, including *Neurospora crassa*, *Magnaporthe oryzae*, *Cryptococcus neoformans*, *Histoplasma capsulatum*, *Ophiostoma* spp. and *Aspergillus* spp. (reviewed by Cogoni, 2001; Nakayashiki *et al*., 2005; Nakayashiki and Nguyen, 2008). Inverted repeat transgenes (IRTs) containing sequences corresponding to the mycotoxin regulatory gene *TRI6* were first shown to inhibit DON production through RNA silencing in *G. zeae* (McDonald *et al*., 2005).

The aims of this work were twofold: first, to test the efficacy of an RNA interfering construct based on IRT targeted at the trichothecene biosynthesis gene *TRI6* of *F. culmorum* and, second, to evaluate whether altered trichothecene biosynthesis by silenced transformants had an effect on the aggressiveness of this CFR-inducing pathogen towards durum wheat seedlings.

RESULTS

Preparation of *F. culmorum* transformants with the *TRI6* IRT

Co-transformation of MCf 21 wild-type and MCf 21 nit1 with the *TRI6* IRT silencing vector pLRM13 and the hygromycin-carrying plasmid pUCH2-8 resulted in over 50 hygromycin B-resistant transformants, among which eight and seven, respectively, were chosen for further analysis. Southern analysis with a *TRI6* probe revealed a variety of integration patterns of the *TRI6* IRT (Figs 1 and 2 and Supporting Information). These included no *TRI6* IRT integrations (i.e. # 282, where only the hygromycin B resistance-carrying vector pUCH2-8 was integrated) to multiple *TRI6* IRT integrations (i.e. # 111). However, many unpredicted bands were observed in our *Nco*I, *Hind*III and *Eco*RI/*Xba*I digests (Figs S1–S4, see Supporting Information). These can be explained by intermolecular recombination between IRTs during the transformation process. Using predictions based on such a model (Fig. S5, see Supporting Information), integration patterns were determined for the 15 transformants (Tables 1 and 2). Thus, transformants were found to have: (i) no IRT, (ii) an 'ideal' IRT, (iii) a promoter-bound IRT (i.e. containing the IRT between opposing *gpdA* promoter sequences), (iv) a terminator-bound IRT (i.e. containing the IRT between diverging *trpC* terminators) or any of the types of IRTs in various combinations (Table 1 and Supporting Information).

Gene expression in *TRI6* IRT *F. culmorum* transformants

Relative to control strains, *TRI6* amplification levels appeared to increase in most of the transformants (significantly higher for strains # 252, 260 and 106), except for # 282 (no pLRM13 integration) and # 260 (Figs 1C and 2C). Active silencing of *TRI6* should result in degradation of the *TRI6*-specific mRNA transcripts. During this process, truncated transcripts of several sizes are generated that may be detected by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) applications that usually amplify 100–150-bp-long fragments. Northern blots carried out with a 478-bp-long *TRI6* probe added further evidence to this hypothesis. Most strains revealed a *TRI6* smear

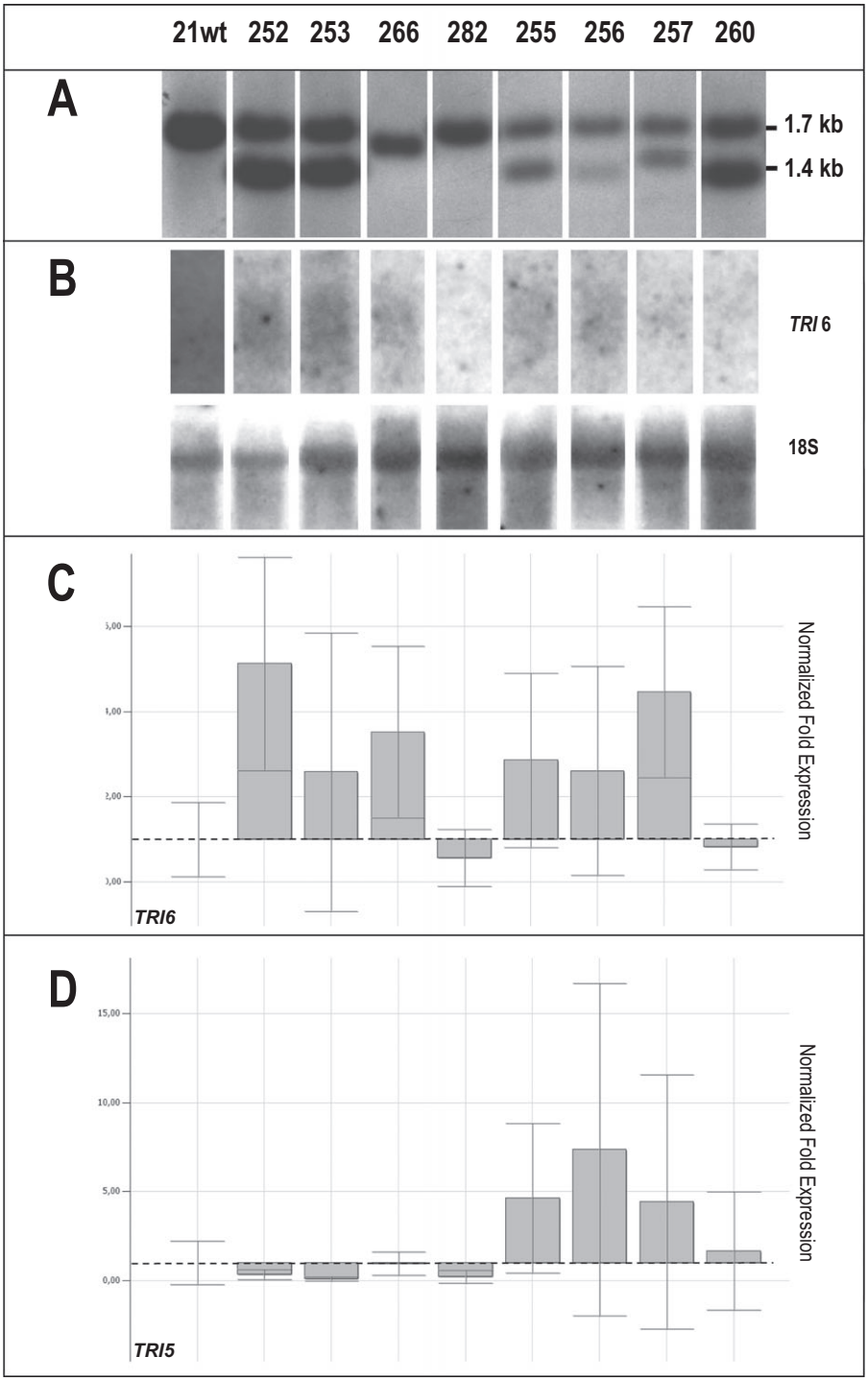

Fig. 1 Characterization and gene expression analysis of *Fusarium culmorum* MCf 21 wild-type (21 wt) and eight independent *TRI6* inverted repeat transgene (IRT) transformants (# 252, 253, 266, 282, 255, 256, 257, 260). (A) Southern blot analysis using the 'sense' *TRI6* IRT fragment of vector pLRM13 to probe *Nco*I/*Hind*III-digested genomic DNAs, resulting in a 1.7-kb band for the endogenous *TRI6* gene and a 1.4-kb band in the case of *TRI6* IRT integration. (B) Northern blot analysis of *TRI6* expression using a 478-bp-long PCR-amplified *TRI6*-specific probe and control hybridization with the 18S probe. (C, D) Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) on expression of the *TRI6* gene (C) and the *TRI5* gene (D); normalization was achieved by comparing the expression of housekeeping genes β-tubulin and 18S; broken line indicates MCf21 21 wt expression level; all other values correspond to increased or decreased gene expression compared with the latter.

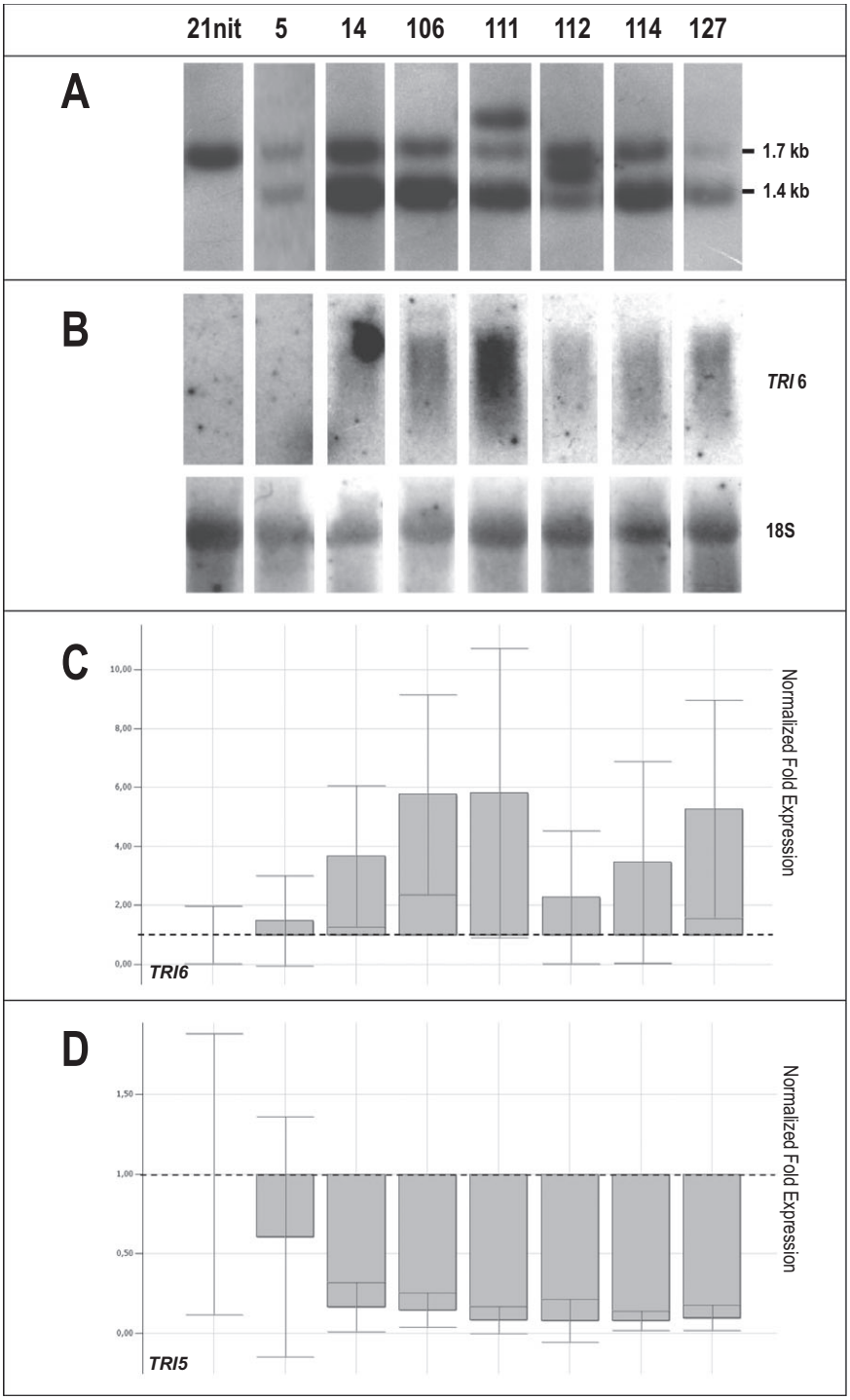

Fig. 2 Characterization and gene expression analysis of *Fusarium culmorum* MCf 21 nit1 (21 nit) and seven independent *TRI6* inverted repeat transgene (IRT) transformants (# 5, 14, 106, 111, 112, 114, 127). (A) Southern blot analysis using the 'sense' *TRI6* IRT fragment of vector pLRM13 to probe *Nco*I/*Hind*III-digested genomic DNAs, resulting in a 1.7-kb band for the endogenous *TRI6* gene and a 1.4-kb band in the case of *TRI6* IRT integration. (B) Northern blot analysis of *TRI6* expression using a 478-bp-long PCR-amplified *TRI6*-specific probe and control hybridization with the 18S probe. (C, D) Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) on expression of the *TRI6* gene (C) and the *TRI5* gene (D). Normalization was achieved by comparing the expression of housekeeping genes β-tubulin and 18S; broken line indicates MCf21 21 nit 1 expression level; all other values correspond to increased or decreased gene expression compared with the latter.

Table 1 *Fusarium culmorum* MCf 21 wild-type and eight independent *TRI6* inverted repeat transgene (IRT) co-transformants. Section 1 contains data from Southern analysis, suggesting the presence of several IRT types which are defined here as follows: ideal IRT, a *TRI6* IRT expressed by a single promoter and bound by a single terminator; promoter-bound IRT, a terminator-less *TRI6* IRT driven by dual *gpdA* promoters; terminator-bound IRT, a promoter-less *TRI6* IRT bound on either side by *trpC* terminators. Section 2 shows the production of deoxynivalenol (DON) and its acetylated derivatives (3-AcDON and 15-AcDON) and the severity of foot and crown rot in durum wheat.

	1					2			
Strain	Ideal IRT	Promoter-bound IRT	Terminator-bound IRT	Various *TRI6* fragments	Northern *TRI6* smear	Total DON (ng/mL)*	Difference of means	Disease index (0–100)†	Difference of means
21 wt	0	0	0	0	No	878.1 ± 41.6		69.2 ± 4.5	
252	0	1	0	0	Yes	49.0 ± 22.0	–829.1	30.4 ± 6.7**‡	–38.75
253	0	1	0	0	Yes	0	–878.1	44.6 ± 5.4**	–24.58
266	0	0	0	1§	Yes	27.6 ± 12.6	–850.5	37.1 ± 5.3**	–32.08
282	0	0	0	0	No	48.2 ± 21.6	–829.9	69.6 ± 5.1	0.42
255	1	0	0	0	Yes	12 783 ± 830**	11 905.0	97.9 ± 1.6**	28.75
256	1	0	0	0	Yes	24 341 ± 5400**	23 463.4	99.6 ± 0.4**	30.42
257	1	0	0	0	Yes	12 473 ± 3 231**	11 594.9	100**	30.83
260	0	0	1	0	No	12 008 ± 2 637**	11 129.7	98.3 ± 1.7**	29.17

*Pooled data from two independent experiments ($P = 0.468$) are expressed as the sum of DON and its acetylated derivatives (ng/mL of culture broth) ± mean SE after 2 weeks of growth in Vogel's medium.

†Pooled data from two independent experiments, carried out in 2008 and 2009 using wheat cultivars Prometeo and Solex, respectively ($P = 0.799$), are expressed as disease incidence (McKinney index 0–100; McKinney, 1923) ± mean SE 3 weeks after inoculation and sowing under glasshouse conditions.

‡Values in each column followed by two asterisks are significantly different from the MCf 21 wild-type control by Dunnett's test ($P < 0.001$).

§Transformant 266 represents an insertion of the IRT at the native *TRI6* locus.

Table 2 *Fusarium culmorum* MCf 21 nit1 and seven independent *TRI6* inverted repeat transgene (IRT) co-transformants. Section 1 contains data from Southern analysis, suggesting the presence of several IRT types which are defined here as follows: ideal IRT, a *TRI6* IRT expressed by a single promoter and bound by a single terminator; promoter-bound IRT, a terminator-less *TRI6* IRT driven by dual *gpdA* promoters; terminator-bound IRT, a promoter-less *TRI6* IRT bound on either side by *trpC* terminators. As a result of the complexity of the integration patterns, it was not always possible to predict the exact type and number of IRT (i.e. 0–1, 1–2). Section 2 shows the production of deoxynivalenol (DON) and its acetylated derivatives (3-AcDON and 15-AcDON) and the severity of foot and crown rot in durum wheat.

	1					2			
Strain	Ideal IRT	Promoter-bound IRT	Terminator-bound IRT	Various *TRI6* fragments	Northern *TRI6* smear	Total DON (ng/mL)*	Difference of means	Disease index (0–100)†	Difference of means
21 nit	0	0	0	0	No	898.1 ± 58.4		97.1 ± 1.9	
5	0	0	1	?	No	890.6 ± 34.8	–7.5	97.9 ± 2.1	0.83
14	0–1	2	2	?	Yes	0**‡	–898.1	21.3 ± 4.9**	–75.75
106	1	1	2	0	Yes	0**	–898.1	33.3 ± 6.1**	–63.75
111	1	1	2	?	Yes	0**	–898.1	25.4 ± 3.2**	–71.67
112	0–1	0–1	0	1–2	Yes	96.1 ± 43.1**	–801.9	22.1 ± 7.6**	–75.00
114	1–2	0	0	0	Yes	0**	–898.1	40.4 ± 5.2**	–56.67
127	1–2	0	1–2	0	Yes	0**	–898.1	20.0 ± 5.3**	–77.08

*Pooled data from two independent experiments ($P = 0.171$) are expressed as the sum of DON and its acetylated derivatives (ng/mL of culture broth) ± mean SE after 2 weeks of growth in Vogel's medium.

†Pooled data from two independent experiments, carried out in 2008 and 2009 using wheat cultivars Prometeo and Solex, respectively ($P = 0.626$), are expressed as disease incidence (McKinney index 0–100; McKinney, 1923) ± mean SE 3 weeks after inoculation and sowing under glasshouse conditions.

‡Values in each column followed by two asterisks are significantly different from the MCf 21 nit1 control by Dunnett's test ($P < 0.001$).

whose intensity typically correlated with the qRT-PCR amplification signal (Figs 1B and 2B). In transformants # 282, 260 and 5, no hybridization signal was detected in Northern blots, correlating with their very low *TRI6* expression levels detected by qRT-PCR analysis.

Semi-quantitative RT-PCR with primers TRI6probeF1 and TRI6probeR2 (Table 3) gave rise to a faint amplification signal of 478 bp for all silenced transformants, except # 255, 256, 257 and 260, which presented a signal comparable with that shown by the reference strain MCf 21 wild-type (Fig. S6, see Supporting Information).

The effect of the *TRI6* silencing construct on the expression of *TRI5* was evaluated by qRT-PCR. The results were fairly congruent with the level of *TRI6* expression: transformants

Table 3 Primers used throughout this study.

Application	Primer sequence	Amplicon size (bp)
Vector construction		
TRI6senseF-*Nco*I*	5′-CCT*CCATGG*TCGTGTTGCGTCTCCCGATCC-3′	570
TRI6senseR-*Asc*I	5′-GCA*GGCGCGCC*ACCCTGCTAAAGACCCTCAG-3′	
TRI6antisenseF-*Not*I	5′-CTTTGAT*GCGGCCGC*GTCTCCCGATCCTG-3′	570
TRI6antisenseR-*Bam*HI	5′-GCATG*GGATCC*ACCCTGCTAAAGACCCTCAG-3′	
Real-time qRT-PCR		
TRI5forb	5′-ACCCTCAATTCCTTCGTCGTATG-3′	141
TRI5revb	5′-CCCAAACCATCCAGTTCTCCATC-3′	
TRI6forb	5′-TTATCGCCCTTCCCACCTTCAC-3′	90
TRI6revb	5′-TAAAGTCCCGTCCGCTCTCAAAG-3′	
18Sforb	5′-TTGACCCGTTCGGCACCTTAC-3′	75
18Srevb	5′-AAGTTTCAGCCTTGCGACCATAC-3′	
tubfor	5′-TTCCAAATCACCCACTCTC-3′	104
tubrev	5′-GAAAGTTGCCATCATACGG-3′	
Probes		
TRI6probeF1	5′-GACTTTGACAACTTCCCCACAT-3′	478
TRI6probeR2	5′-AGTGATCTCGCATGTTATCCAC-3′	
18S-Forward	5′-CCTTAACGAGGAACAATTGGAG-3′	514
18S-Reverse	5′-CCCTAGTCGGCATAGTTTATGG-3′	
Restriction analysis of *TRI4–TRI6–TRI5* cluster		
H-11 For4	5′-GTGAACTTCGCGGGCGTTTACTC-3′	5408
H-11 Rev13	5′-TCCGGCTTGAAGGTCGTCAAAAT-3′	

*Restriction sites are given in italic.

252, 253, 266, 5, 14, 106, 111, 112, 114 and 127, which showed more or less strongly smeared *TRI6*-specific signals in Northern blots, showed correspondingly more or less decreased *TRI5* expression compared with the recipient strains MCf 21 wild-type and MCf 21 nit1, respectively (Figs 1D and 2D).

Transformants # 255, 256 and 257 revealed a completely different pattern, displaying a significantly increased *TRI5* expression compared with MCf 21 wild-type. Transformant # 260 showed almost the same level of *TRI*6 expression as MCf21 wild-type and a slightly, but not significantly, increased *TRI*5 expression (Fig. 1D).

Production of trichothecenes *in vitro* by transformed *F. culmorum* strains

Based on a previous report by Boutigny *et al*. (2009), who demonstrated that strain MCf 21 wild-type is able to produce predominantly 3-AcDON and, to a lesser extent, DON when grown in liquid synthetic medium, we focused our high-performance liquid chromatography-mass spectrometry (HPLC-MS) analysis on DON and its acetylated derivatives 3-AcDON and 15-AcDON. In a preliminary experiment, the average (mean of six replicates ± SD) production of type B trichothecenes (ng/mL culture filtrate) by this strain was 212.6 ± 74.2 DON, 523.0 ± 98.9 3-AcDON and 16.3 ± 3.9 15-AcDON after 14 days of growth in Vogel's medium at 25 °C. This pattern of distribution was confirmed in subsequent analyses.

In Tables 1 and 2, pooled data from two independent experiments carried out with MCf 21 wild-type-derived transformants, or with MCf 21 nit1-derived transformants (*P* values between two equivalent experiments of 0.468 and 0.171, respectively), are expressed as the sum of DON, 3-AcDON and 15-AcDON in ng/mL culture filtrate ± SE. Regression was linear over the tested concentration range (5–250 ng/mL), with an average correlation coefficient R^2 of 0.9982 (±SD = 0.001), calculated from five calibration curves.

In MCf 21 wild-type-derived transformants # 252 and 253, the production of type B trichothecenes was reduced by 95% and 100%, respectively (Table 1). In transformants # 266 (homologous recombination with the endogenous *TRI6* gene) and 282 (no apparent *TRI6* IRT integration), type B trichothecene production was reduced to 3.1% and 5.4% with respect to the recipient strain. Interestingly, transformants # 255, 256, 257 and 260—which showed increased *TRI5* expression—produced 14–28-fold more trichothecenes than the MCf 21 wild-type strain, corresponding to average concentrations ranging from 12 008 ± 2637 to 24 341 ± 5400 ng/mL of culture filtrate (Table 1).

Among *TRI6* IRT transformants derived from the nitrate reductase mutant MCf 21 nit1, only transformant # 5 did not differ from the recipient strain for the production of type B trichothecenes, whereas, in transformant 112, trichothecene production was reduced by approximately 90% (Table 2). In all other *TRI6* IRT transformants (i.e. # 14, 106, 111, 114 and 127), the production of type B trichothecenes was completely suppressed (Table 2).

Virulence of *TRI6* IRT transformants on durum wheat

In order to ascertain whether altered trichothecene production by *TRI6* IRT transformants affected their virulence, inoculation on durum wheat was carried out with both MCf 21 wild-type- and MCf 21 nit1-derived transformants using two different cultivars (Prometeo and Solex; *P* values between two equivalent experiments of 0.799 and 0.626, respectively). Indeed, among the 15 transformants tested, only # 282 did not differ significantly from the recipient strain MCf 21 wild-type, despite a significant reduction in its DON production (Table 1), whereas # 5 produced the same amount of trichothecenes and displayed the same virulence as the nontransformed strain MCf 21 nit1 (Table 2). In all other transformants, significant changes (i.e. increase or decrease) in type B trichothecene production reflected corresponding differences in virulence (Tables 1 and 2).

DISCUSSION

Together with *F. graminearum* and *Fusarium avenaceum*, the plant pathogenic fungus *F. culmorum* is ranked among the three most important wheat head blight pathogens on a global scale (Wagacha and Muthomi, 2007). Although considerable efforts have been devoted by the research community to dissect the pathogenicity and mycotoxin production of *G. zeae* (Goswami and Kistler, 2004), the pathogenicity determinants of *F. culmorum* and the role played by trichothecenes in the severity of CFR disease on wheat by this pathogen are still poorly understood. Although DON is considered as an important virulence factor in *F. culmorum* head blight disease (McCormick, 2003; Wagacha and Muthomi, 2007), little is known of the role of DON in CFR. Therefore, the main objective of this work was to elucidate the relationship between DON production by *F. culmorum* and its aggressiveness towards durum wheat seedlings.

To suppress DON production through an RNA silencing approach in *F. culmorum*, we generated an RNA interfering construct targeted at the endogenous regulatory gene *TRI6*. This gene encodes a Cys_2-His_2-type transcription factor binding to the promoter DNA sequence motif TNAGGCCT of the trichodiene synthase-encoding gene *TRI5* (Hohn *et al*., 1999). The same approach has been applied already to *G. zeae* by McDonald *et al*. (2005), who reported four *TRI6* IRT transformants with reduced DON production and decreased virulence on wheat heads.

In this study, which represents the first report of the suppression of gene expression via RNA silencing in *F. culmorum*, a series of *TRI6* IRT transformants was obtained from the highly virulent strain MCf 21 wild-type (syn. INRA 117) and its nitrate reductase-deficient mutant MCf 21 nit1.

The main achievement of this report is that, with only a few exceptions, a tight relationship exists between trichothecene production in culture and virulence on durum wheat seedlings. This was demonstrated to hold true in both directions, i.e. most of the transformants with a reduced *TRI5* expression signal as measured by qRT-PCR also displayed reduced DON production and virulence, whereas four trichothecene-overproducing transformants showed increased *TRI5* signals in qRT-PCR experiments and significantly higher virulence on durum wheat compared with the recipient MfC 21 wild-type strain.

During the course of the experiments, the level of expression of the target gene appeared to be unpredictable: almost all the transformants displayed an apparently increased *TRI6* expression by both Northern analysis and qRT-PCR compared with the respective recipient strain. We interpreted this puzzling result as the accumulation of silencing IRTs consequent to the adoption of the constitutive promoter *gpdA*. Truncated transcripts of several sizes are generated by the silencing complex, before passing on to complete fragmentation and elimination from the cell. This assumption was substantiated by the appearance of a smeared hybridization signal in Northern blots. Silenced RNAs may act as a substrate for qRT-PCR, given the small size—i.e. 90 bp—of the expected amplicon, hence the appearance of an increased level of *TRI6*-specific amplification in the transformants compared with the recipient strain. Indeed, RT-PCR experiments targeted at a 478-bp amplicon showed a fainter signal in most silenced transformants compared with the MCf 21 wild-type (see Supporting Information). Moreover, in most MCf 21 nit1-derived transformants, stronger *TRI6*-hybridizing smears and *TRI6*-specific amplification signals corresponded to a reduced expression of the trichodiene synthase gene *TRI5*, suggesting that *TRI6* silencing and subsequent *TRI5* suppression indeed occurred.

Among the MCf 21 wild-type-derived transformants, four strains were unexpectedly obtained with significantly increased *TRI5* expression. These strains were able to produce an impressively large amount of DON (i.e. 14–28-fold more than that of the wild-type strain, corresponding to 12–24 μg/mL of culture filtrate). We first speculated that the *gpdA* promoter used in the *TRI6* IRT could have recombined with the endogenous *TRI6* locus, generating a series of *gpdA*-*TRI6* recombinant strains. However, Southern analysis with a *TRI6* probe and comparative restriction analysis of the *TRI4–TRI6–TRI5* locus (see Supporting Information) confirmed that the endogenous *TRI6* locus was intact. These superproducing transformants are now being analysed at the proteome level, as the *gpdA* promoter could have recombined with a new positive regulatory gene, possibly suggesting alternative avenues in the regulation of trichothecene biosynthetic genes.

An additional finding of Southern analysis was that recombination between IRTs may occur quite frequently on integration into the *F. culmorum* genome. The silenced strains displayed a promoter-bound IRT, a terminator-bound IRT or an 'ideal' IRT with additional undetermined IRT fragments. Surprisingly, the overexpressing transformants appear to be among the simplest

integrations, showing evidence for only one IRT. Moreover, the level of silencing did not correlate with the copy number of the IRT construct integrated into the genome: MCf 21 nit1-derived transformants having integrated single or multiple copies of pLMR13 showed similarly reduced *TRI5* expression levels and DON production. In one case (transformant # 5), *TRI6* IRT integration did not generate any apparent modification in type B trichothecene biosynthesis. This is not surprising, as the same situation has been reported with *Ophiostoma floccosum* by Tanguay *et al*. (2006) and *Aspergillus fumigatus* by Henry *et al*. (2007), who highlighted the disadvantages of the RNA silencing approach compared with classical gene disruption by gene replacement. Thus, in the present work, we show, for the first time, that a functional RNA silencing pathway may be active in the phytopathogenic fungus *F. culmorum*, but, at the same time, we provide evidence for unpredictable patterns of IRT integration and silencing efficiency.

Our results demonstrate that, together with pectolytic enzymes (Aleandri *et al*., 2007; Kang and Buchenauer, 2002), type B trichothecenes play an important role as virulence factors in the CFR disease of durum wheat caused by *F. culmorum*, thus confirming this biosynthetic pathway as a suitable molecular target for the development of new CFR control tools. Modulation of the accumulation of trichothecenes in the plant tissues opens up new perspectives for the development of cereal varieties at reduced 'mycotoxin risk', through the action of plant endogenous compounds (Boutigny *et al*., 2008), or by delivering gene-targeted RNA silencing triggers into the host plant (McDonald *et al*., 2005). Alternatively, a biocontrol approach could be envisaged that uses silenced atoxigenic mutants with low or null virulence. The latter strategy has been applied successfully to control aflatoxin-producing *Aspergillus flavus* through competitive exclusion by atoxigenic isolates (Cotty *et al*., 2007).

Indubitably, before a biocontrol approach can be conceived, a thorough risk assessment is needed to ensure the stability of the integrated silencing construct and to avoid the possibility of unwanted recombination events involving the constitutive promoter, the *TRI* locus or other regulatory genes acting on the *TRI* cluster. Although our experiments were carried out in the laboratory or in contained facilities, the unpredictable generation of superproducing transformants opens up a new scenario for the definition of a biosafety paradigm in silencing experiments.

EXPERIMENTAL PROCEDURES

Strains and culture conditions

Fungal spores were obtained from: (i) a highly virulent strain of *F. culmorum* (W.G. Smith) Sacc. (strain ISPaVe MCf 21 wild-type; syn. strain INRA 117), isolated in 1989 from triticale grown in Foggia, Apulia, southern Italy; (ii) its nitrate reductase-deficient mutant MCf 21 nit1; and (iii) transformants derived by growing in a medium containing Campbell's V8 Vegetable juice (Giobbe *et al*., 2007) for 7 days at 25 °C with agitation (150 rpm). Cultures were filtered and conidia were collected by centrifugation. Spores were then resuspended in 10% glycerol at 10^6/mL and stored at −80 °C until needed.

In liquid culture, strain MCf 21 produces predominantly 3-AcDON and, to a lesser extent, DON (Boutigny *et al*., 2009). To monitor the production of DON, 3-AcDON and 15-AcDON, and the expression of *TRI5* and *TRI6* genes, each strain was used to inoculate 8 mL of Vogel's medium (Vogel, 1956) with spore suspensions to achieve a final concentration of 10^4 conidia/mL. Fungal liquid cultures were incubated in the dark at 25 °C by gentle shaking at 30 rpm. Cultures were performed in triplicate and each experiment was repeated at least twice. Cultures were sampled after 7 days of incubation for RT-PCR and qRT-PCR (mycelium) and after 14 days for HPLC-MS analysis (filtrates).

Construction of the silencing vector

The primers used in vector construction are listed in Table 3. A 570-bp fragment of the *TRI6* gene of *F. culmorum* (GenBank accession number AF480836) was amplified from genomic DNA, using a forward primer containing a *Nco*I site (TRI6senseF-*Nco*I) and a reverse primer containing an *Asc*I site (TRI6senseR-*Asc*I). The PCR product was digested and ligated into the *NcoI*/*Asc*I sites of pTMH44.2 (McDonald *et al*., 2005) to produce pTRM20. A similar *TRI6* fragment of 570 bp was amplified by PCR, using a forward primer containing a *Not*I site (TRI6antisenseF-*Not*I) and a reverse primer containing a *Bam*HI site (TRI6antisenseR-*Bam*HI). This PCR product was digested and ligated in reverse orientation into the *NotI*/*Bam*HI sites in pTRM20 to obtain pLRM13 (Fig. 3).

Transformation experiments

Fungal protoplasts were isolated from germinated microconidia. For this purpose, 10–12 plates containing potato dextrose agar (PDA), covered with a sheet of sterile cellophane, were each inoculated with 10^6 conidia of *F. culmorum* MCf 21 wild-type or MCf 21 nit1, and incubated at 25 °C for 16–18 h. Young thalli were then harvested with a sterile spatula and transferred into 20 mL of digestion solution consisting of 1.2 M $MgSO_4$, pH 5.8, containing 10 mg/mL of lysing enzymes (L1492, Sigma-Aldrich, St. Louis, MO, USA). After 3–3.5 h of incubation at room temperature and shaking at about 50 rpm, protoplasts were purified according to Langin *et al*. (1990) and then co-transformed with 10 μg of the silencing vector pLRM13 and 10 μg of the hygromycin B resistance-carrying vector pUCH2-8 (Alexander *et al*., 1998) as described by Hua-Van *et al*. (2001). Aliquots of 50 μL of the transformation mixture were transferred into 4–5 mL of

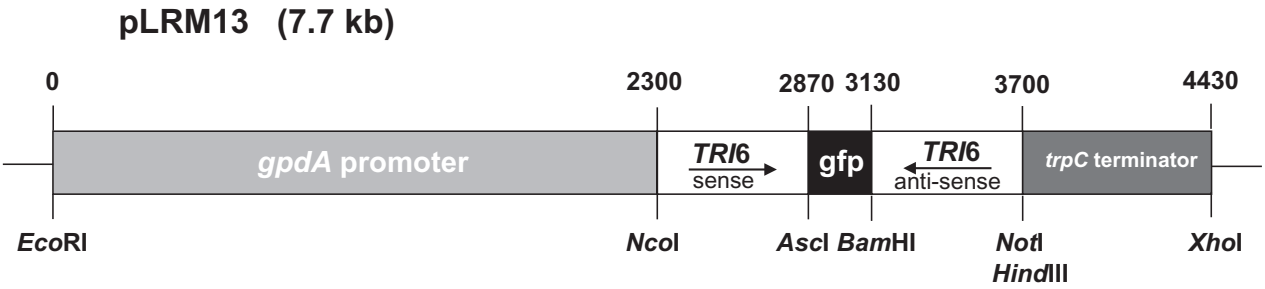

Fig. 3 Schematic representation of *Fusarium culmorum TRI6* inverted repeat transgene (IRT) construct. Light grey, *gpdA* promoter of *Aspergillus nidulans*; dark grey, *trpC* terminator of *A. nidulans*; black, *gfp* spacer element. A 570-bp fragment of the *F. culmorum TRI6* gene was placed in sense and antisense directions on the two sides of the *gfp* spacer element in the vector pTMH44.2, generating vector pLRM13.

preheated (37 °C) top agar [7.3 mM KH_2PO_4, 2 mM $MgSO_4$, 6.5 mM KCl, 36 μM $FeSO_4$, 23.5 mM $NaNO_3$, 2% (w/v) glucose, 0.8% (w/v) technical agar] and plated onto Petri dishes containing PDAS H50 medium [potato dextrose agar containing 20% (w/v) sucrose and 50 μg/mL hygromycin B]. After incubation at 25 °C for 5–7 days, hygromycin B-resistant transformants were selected for the integration of pUCH2-8 and purified by monosporic culturing, followed by Southern blot analysis to control the correctness of pLRM13 integration events.

Southern analysis of transformants

Selection for hygromycin B-resistant co-transformants carrying the pLRM13 vector was performed by Southern blot analysis. Genomic DNA was purified from lyophilized mycelium by a miniprep method described previously (Migheli *et al*., 1996). Ten micrograms of genomic DNA were digested with 50 U each of *Hind*III, *Nco*I, *Nco*I/*Hind*III and *Eco*RI/*Xba*I (New England Biolabs Inc., Ipswich, MA, USA) at 37 °C for 15–18 h, separated by 1.0% agarose gel electrophoresis and transferred onto nylon membranes (Hybond-N, Amersham Biosciences, Uppsala, Sweden) using a vacuum blotter (Vacuum Blotter Model 785, Bio-Rad Laboratories, Milan, Italy) according to standard procedures (Sambrook *et al*., 1989).

The blots were then hybridized with a 570-bp probe, complementary to the *TRI6* sense fragment of the vector pLRM13. One microgram of pLRM13 (Fig. 3) was digested with 20 U each of *Asc*I and *Nco*I in a volume of 100 μL at 37 °C for 3 h; digested DNA was precipitated and then loaded onto a 2.0% agarose gel. The 570-bp DNA band was recovered with a sterile scalpel directly from the gel, followed by a silica column-based purification step using the Jet Quick Gel extraction spin kit (Genomed GmbH, Löhne, Germany). Labelling, hybridization and detection reactions were carried out with the Gene Images Random Prime Labelling® and Gene Images CDP Star Detection® modules (Amersham Biosciences Europe GmbH, C. Monzese, Italy), as described in the manufacturer's protocols. Briefly, the modules work with a chemiluminescent signal, incorporating F1-dUTP molecules in the future probe by a PCR approach. After this labelling step, the DNA probe was applied in an overnight hybridization step at 55–60 °C. Following stringency washes and blocking of nonspecific sites on the membrane, the chemiluminescent signal was activated with an alkaline phosphatase conjugate after incubation with the CDP Star detection substrate. The membranes were exposed to X-ray radiographic films at room temperature for 1–2 h and then developed.

Sequencing and restriction analysis of the *TRI4–TRI6–TRI5* locus in *F. culmorum*

To evaluate whether the endogenous *TRI6* locus could be affected by the integration of the silencing vector, the 7.6-kb region spanning the complete *TRI4–TRI6–TRI5* genes in the *F. culmorum* MCf 21 nit1 strain was sequenced by a PCR-based approach. Fifteen primer pairs generating overlapping amplicons of 500–700 bp in this region were designed based on the *G. zeae* H-11 *TRI* locus (GenBank accession number AF336366). PCR was carried out as follows: 1 × Platinum® *Pfx* DNA Polymerase amplification buffer (Invitrogen, Invitrogen S.R.L., San Giuliano Milanese (MI), Italy), 1 mM $MgSO_4$, 0.2 mM deoxynucleoside triphosphates (dNTPs), 0.5 μM of forward and reverse primers, 1.0 U of Platinum® *Pfx* DNA Polymerase (Invitrogen) and 300 ng of template DNA were mixed in a final volume of 50 μL and denatured at 95 °C for 3 min, followed by 32 cycles consisting of denaturation at 95 °C for 30 s, annealing at 60 °C for 45 s and elongation at 68 °C for 1 min, concluded by a final elongation step at 68 °C for 7 min.

Amplification products were examined on agarose gel for their integrity and specificity, purified and then sent for sequencing to BMR Genomics srl (Padua, Italy). Every amplification product was sequenced in both forward and reverse directions. Quality control of the single sequences and their assembly to the complete sequence were carried out with Sequence Scanner v1.0 and CLC Sequence Viewer 6 software. GenBank accession number: BankIT 1344398, HM131844 (Fig. S7, see Supporting Information).

A comparative restriction analysis of the 5.4-kb region, including the final stretch of the *TRI4* gene, the complete *TRI6* gene, the initial stretch of the *TRI5* gene and the two intergenic regions comprised between them, was carried out with the *F. culmorum* MCf 21 wild-type, MCf 21 nit1 and 15 derived IRT transformants. The 5.4-kb region was amplified with the primers H-11For4 and H-11Rev13 (Table 3) applying the Expand Long Template PCR System Kit (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturer's protocol. The following restriction enzyme combinations were tested: *Hind*III, *Nco*I, *Hind*III/*Nco*I, and *Eco*RI/*Xba*I (New England Biolabs).

RNA purification and cDNA synthesis

Total RNA was extracted using the PureLink™ Micro-to-Midi total RNA purification system (Invitrogen) according to the manufacturer's instructions for RNA purification from plant tissue. A DNAse treatment to remove genomic DNA contamination from the samples was performed using the 'RQ1 RNase-freeDNase' (Promega, Madison, WI, USA). The concentration of RNA was determined spectrophotometrically using GeneQuant (Amersham Biosciences). For cDNA synthesis, 0.5 μg of RNA were retrotranscribed with the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories). The cDNA was either used directly for real-time qRT-PCR or stored at 4 °C.

qRT-PCR analysis

The ICycler iQ system coupled to the SYBR® Green Kit (Bio-Rad Laboratories) was used for qRT-PCR. The real-time qRT-PCR efficiencies were determined for each gene by measuring C_T to a specific threshold (Walker, 2002) for five serial 1 : 2 dilutions of the *F. culmorum* strain MCf 21 wild-type cDNA. Primers applied for the amplification of *TRI5*, *TRI6*, 18S rRNA and β-tubulin in the qRT-PCR approach had efficiencies of 98.7%, 96.0%, 90.7% and 96.4%, respectively. The specificity of each primer set was examined and the amplification of a specific transcript was confirmed by the appearance of a single peak in the melting curve analysis following completion of the amplification reaction. The relative level of expression of each gene of interest (C_T) was then normalized against the relative gene expression level of the RNA coding for the reference genes β-tubulin and 18S rRNA determined in each sample ($\Delta\Delta C_T$).

Primers (Table 3) were designed using the software Molecular Beacon (Bio-Rad Laboratories). Primers used for the amplification of *TRI5* (TRI5forb/TRI5revb) and *TRI6* (TRI6forb/TRI6revb) were derived from conserved regions in *F. culmorum* (GenBank accession numbers AY130291 and AF480836). Primers used for the amplification of the 18S rRNA gene (18Sforb/18Srevb) were derived from *G. zeae* (accession number AB250414). Primers used for the amplification of the β-tubulin gene (tubfor/tubrev) were derived from *F. culmorum* (accession number EU490256).

The reaction mixtures contained 1 μL of cDNA diluted 1 : 10 (=2.5 ng), 1 μL of each primer (400 nM), 12.5 μL of iQ™ SYBR® Green Supermix and 9.5 μL of Milli-Q H_2O in a final volume of 25 μL.

In all the experiments, appropriate negative controls containing no template were subjected to the same procedure to exclude or detect any possible contamination or carry-over. Each sample was amplified three times for each experiment and all the experiments were repeated at least twice. The programme used included one step at 95 °C for 3 min, and 40 cycles consisting of 95 °C for 10 s and 60 °C for 30 s, followed by gradual heating (0.5 °C every 30 s) from 50 to 85 °C in order to generate the melting curve.

RT-PCR analysis

In a semi-quantitative approach, the cDNA of MCf 21 wild-type and 15 *TRI6* IRT transformants was subjected to RT-PCR analysis, applying primers spanning almost the entire *TRI6* gene (TRI6probeF1/TRI6probeR2; Table 3). One-hundred nanograms of cDNA of each sample were amplified in a reaction volume of 50 μL with 1 × TopTaq™ DNA Polymerase amplification buffer (Qiagen, Qiagen S.p.A., Milan, Italy), 0.2 mM dNTPs, 0.5 μM of forward and reverse primers and 2.0 U of TopTaq™ DNA Polymerase (Qiagen). Different numbers of amplification cycles were tested to ascertain that the PCR products did not represent a saturated signal in the following gel electrophoresis. Samples were denatured at 95 °C for 3 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 48 °C for 45 s and elongation at 72 °C for 1 min, with a final elongation step at 72 °C for 7 min. For all samples, a control reaction was carried out with 18S primers (18S-Forward/18S-Reverse; Table 3).

Northern analysis

For Northern blot hybridization experiments, mycelia of strains MCf 21 wild-type and derived transformants # 252, 253, 255, 256, 257, 260, 266 and 282, as well as MCf 21 nit1 and derived transformants # 5, 14, 106, 111, 112, 114 and 127, were harvested after 7 days of growth in Vogel's medium, as described previously, flash-frozen by dipping in liquid nitrogen, ground to a fine powder and total RNAs were isolated according to the guanidinium-thiocyanate/phenol method (Chomczynski and Sacchi, 1987). Twenty micrograms of total RNA of each sample to be tested were separated on a 1.2% formaldehyde gel and transferred onto nylon membranes (Hybond-N, Amersham Biosciences) using a vacuum blotter (Vacuum Blotter Model 785, Bio-Rad Laboratories) according to standard procedures (Sambrook *et al*., 1989).

Expression levels of the *TRI6* gene were detected for all strains by hybridization with a *TRI6*-specific probe (478 bp). In addition,

a control hybridization was carried out with an 18S rRNA probe to evaluate the basal quantity and quality of the RNA signal. DNA fragments for the future probes were amplified by PCR on the cDNA template of MCf 21 wild-type strain applying the following primers: TRI6probeF1/TRI6probeR2, 18S-Forward/18S-Reverse (Table 3). Labelling, hybridization and detection reactions were carried out with the Gene Images Random Prime Labelling® and Gene Images CDP Star Detection® modules (Amersham Biosciences), as described previously.

HPLC-MS analysis of trichothecenes

Standard mixtures including NIV, DON, 15-AcDON and 3-AcDON were obtained from Sigma Chemicals (St. Louis, MO, USA). The samples (8 mL) were extracted with 5 mL of ethyl acetate. After centrifugation, the organic phase was evaporated to dryness and redissolved in 1 mL of LC-MS mobile phase. An Agilent Technologies (Palo Alto, CA, USA) 1100 series LC-MSD, equipped with a diode-array detector and an autosampler, was used for LC separation. Chromatographic separation was achieved using a Luna C18 column (150 mm $\times$ 2.1 mm, 3 mm; Phenomenex, Castelmaggiore (BO), Italy) fitted with a C18 security guard cartridge. The column temperature was maintained at 40 °C. The mobile phase consisted of Eluent A (water with 0.01% acetic acid) and Eluent B (acetonitrile). The separation was performed in a run time of 20 min under gradient conditions with a flow rate of 0.4 mL/min. MS detection was performed using an Agilent (Palo Alto, CA, USA) G1946 (MSD 1100) single-stage quadrupole instrument equipped with an electrospray atmospheric pressure ionization source. To construct standard curves, stock solutions of B-trichothecene were prepared by dissolution in the standard mixture of 10 mL of acetonitrile (final concentration, 1000 ng/mL). Working solutions of GE-B5 were prepared daily by diluting aliquots of stock solutions with the solvent system (Eluent A), and were used to spike samples. Six different concentrations of B-trichothecene (5, 10, 25, 50, 100 and 250 ng/mL) were obtained by adding appropriate concentrations of working solutions to the solvent system.

Data are reported as mean values $\pm$ SE of three biological replications. Trichothecene yields (sum of DON and its acetylated derivatives 3-AcDON and 15-AcDON) are expressed as ng/mL of culture filtrate. The data were subjected to one-way analysis of variance, followed by multiple comparison by Dunnett's test, using Minitab® for Windows release 12.1.

Pathogenicity assay

A monosporic culture of each *F. culmorum* strain was grown on PDA (Difco, Detroit, MI, USA) at 25 °C with a photoperiod of 12 h for 7 days. Plugs (1.2 cm in diameter) of PDA colonized by the fungus were cut with a sterile cork borer and used as inoculum. One plug was placed in the centre of a plastic sowing pot (diameter, 4.5 cm; capacity, 55 mL) containing sterilized (121 °C for 60 min on two successive days) potting mix (Humin-Substrat N17, Neuhaus, Germany). One seed of durum wheat (*Triticum durum* cvs. Prometeo or Solex, kindly provided by Unità di Ricerca per la Valorizzazione Qualitativa dei Cereali, CRA-QCE, Rome, Italy) was placed onto each PDA plug and covered by sterilized soil. For each treatment, three replicates (10 seeds for each replicate) were used. Pots were watered daily and the average temperature was 25 °C (minimum, 15 °C; maximum, 35 °C). Seedling emergence was checked every 7 days for 3 weeks. After the last survey, the plant height and disease severity were evaluated. The severity of disease was calculated using the McKinney index (McKinney, 1923), which expresses the percentage of the maximum severity of disease (i.e. 100) according to the formula: $I = [\Sigma(c \times f)/n \times N] \times 100$, where c is the disease class, f is the frequency, n is the number of observations and N is the highest value of the empirical scale adopted, estimating the severity of symptoms on the stem. Five classes were set: class 0, healthy stem; class 1, mild browning on the stem; class 2, browning on one half of the stem; class 3, complete browning of the stem; class 4, plant death after emergence or plant not emerged in comparison with the emergence of the uninoculated control treatment.

Each experiment was repeated at least twice. The data were subjected to one-way analysis of variance, followed by multiple comparison by Dunnett's test, using Minitab® for Windows release 12.1.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Southern hybridization analysis of *Fusarium culmorum* MCf 21 wild-type (wt), MCf 21 nit1 (nit) and 15 *TRI6* inverted repeat transgene (IRT) transformants using the 'sense' *TRI6* IRT fragment of vector pLRM13 to probe *Hind*III-digested genomic DNAs; selected molecular weights of the 1 kb plus DNA ladder (Invitrogen) indicate the range of the band pattern.
Fig. S2 Southern hybridization analysis of *Fusarium culmorum* MCf 21 wild-type (wt), MCf 21 nit1 (nit) and 15 *TRI6* inverted repeat transgene (IRT) transformants using the 'sense' *TRI6* IRT fragment of vector pLRM13 to probe *Nco*I-digested genomic DNAs; selected molecular weights of the 1 kb plus DNA ladder (Invitrogen) indicate the range of the band pattern.
Fig. S3 Southern hybridization analysis of *Fusarium culmorum* MCf 21 wild-type (wt), MCf 21 nit1 (nit) and 15 *TRI6* inverted repeat transgene (IRT) transformants using the 'sense' *TRI6* IRT fragment of vector pLRM13 to probe *Hind*III/*Nco*I-digested genomic DNAs; selected molecular weights of the 1 kb plus DNA ladder (Invitrogen) indicate the range of the band pattern.
Fig. S4 Southern hybridization analysis of *Fusarium culmorum* MCf 21 wild-type (wt), MCf 21 nit1 (nit) and 15 *TRI6* inverted repeat transgene (IRT) transformants using the 'sense' *TRI6* IRT fragment of vector pLRM13 to probe *Eco*RI/*Xba*I-digested genomic DNAs; selected molecular weights of the 1 kb plus DNA ladder (Invitrogen) indicate the range of the band pattern.
Fig. S5 Plasmid pLRM13 recombination model. (A) pLRM13. (B) Recombination could occur between two molecules of pLRM13. One possibility is that the plasmids recombine in *Fusarium culmorum* before integration into the genome. A theoretical break point between the forward *TRI6* fragment in one plasmid and the reverse *TRI6* fragment in another plasmid is shown. (C) Expected product after recombination as proposed in (B). The distances between the restriction enzymes of the recombined plasmids allow for band DNA fragment sizes consistent with many of the transformants analysed in this study. Depending on how the recombined plasmid integrates into the genome, a transformant could end up with a 'promoter-bound' inverted repeat transgene (IRT) (left) or a 'terminator-bound' IRT (right). Broken lines represent additional plasmid sequences.
Fig. S6 Analysis of *TRI6* gene expression by semi-quantitative reverse transcription-polymerase chain reaction. cDNA of *Fusarium culmorum* MCf 21 wild-type and of 15 *TRI6* inverted repeat transgene (IRT) transformants was amplified with the primer pair TRI6probeF1/TRI6probeR2 (Table 3). Samples were denatured at 95 °C for 3 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 48 °C for 45 s and elongation at 72 °C for 1 min; final elongation was at 72 °C for 7 min. A control reaction was carried out with 18S primers (18S-Forward/18S-Reverse; Table 3).
Fig. S7 Alignment of sequences of the *TRI4–TRI6–TRI5* locus for *Fusarium culmorum* MCf 21 wild-type (HM131844) and *Gibberella zeae* H-11 (AF336366).

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