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Fusarium culmorum Tri genes and barley *HvUGT13248* gene transcription in infected barley cultivars

*Zuzana Faltusová[1], Kateřina Vaculová[2], Jozef Pavel[1], Ilona Svobodová[2], Jana Hajšlová[3], Jaroslava Ovesná[1]**

[1]Crop Research Institute, Prague, Czech Republic; [2]Agrotest Fyto, Ltd., Kroměříž, Czech Republic; [3]Institute of Chemical Technology Prague, Prague, Czech Republic

**Corresponding author: ovesna@vurv.cz*

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Abstract: The transcription activities of genes somehow associated with the mycotoxin deoxynivalenol (DON) biosynthesis, namely *Fusarium Tri* genes, and the barley gene coding for UDP-glycosyltransferase (HvUGT13248) on different genetic backgrounds were compared. Determining the amount of the pathogen DNA was used as a useful tool for evaluating the infestation of barley cultivars. Amounts of the pathogen DNA differed in six barley cultivars infected by *F. culmorum*. Transcription of *HvUGT13248* was related to DON content in the samples. Low pathogenic infection and low DON content were accompanied by increased *Fusarium Tri10* transcription in resistant cv. Amulet. This finding confirmed our recent results and makes us propose using this change as a possible marker of barley resistance against *Fusarium*.

Keywords: barley; UDP-glycosyltransferase; RT qPCR; DON biosynthesis

Phytopathogenic fungi of *Fusarium* species affect cereals not only by development of Fusarium head blight (FHB) after infection but also they significantly decrease grain yield and degrade grain quality due to its contamination by mycotoxins. The most important contamination in cereals is from trichothecene mycotoxins (Foroud & Eudes 2009), which have been known to be hazardous to human and animal health for many years (Rocha *et al.* 2005). In addition, the trichothecene mycotoxin deoxynivalenol (DON) is one of the *F. graminearum* gene transcripts which have been identified that are likely to be involved in barley infection (Güldener *et al.* 2006).

The biosynthetic pathway of trichothecene has been mapped, and the so-called *Tri* genes encoding enzymes involved in this pathway have been identified (Boutigny *et al.* 2009). As summarised by Merhej *et al.* (2012), 15 *Tri* genes are located at 3 different loci in the genome of *F. graminearum*: 12 genes are clustered and form the core *Tri* cluster locus, a two-gene cluster *Tri1–Tri16* is located at another locus, and the unique gene *Tri101* has been identified at a third location. *Tri6* and *Tri10* have been identified as genes regulating other *Tri* genes (Peplow *et al.* 2003). Although it is not yet clear how the Tri6 and Tri10 transcription factors are activated to regulate the expression of other *Tri* genes, cAMP signalling or three MAP kinase pathways are likely involved in transducing extracellular signal to regulate *Tri* gene expression via these transcription factors (Jiang *et al.* 2016).

Resistance of plants to *Fusarium* infection includes resistance based on chemical modification of trichothecene mycotoxins and resistance based on inhibition of trichothecene biosynthesis (Foroud & Eudes 2009). Among others, the enzyme UDP glycosyltransferase, which mediates transformation of DON

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mycotoxin to less toxic deoxynivalenol-3-*O*-glucoside (D3G), has been described. This enzyme is encoded by the gene *HvUGT13248* in barley (Schweiger *et al.* 2010) which has also been identified as suitable for transformation. Transgenic wheat expressing a barley UDP-glucosyltransferase (HvUGT13248) exhibited significantly greater resistance to the spread of disease in the spike compared to non-transformed controls (Li *et al.* 2015). Evaluation of crop resistance to an attack by pathogens is essential for the selection and breeding of resistant cultivars. Head blight rating and DON content of grain were significantly correlated in field experiments involving winter rye infected by *F. culmorum* (Gang *et al.* 1998) and wheat infected by *F. graminearum* (Bai *et al.* 2001). In the case of barley, symptomatic evaluation of resistance to *Fusarium* is even more complicated than in wheat, and no correlation between FHB severity and DON accumulation was confirmed in any of those cases summarized by Chrpová *et al.* (2011). It is therefore necessary to find a suitable tool for evaluating infestation in plants in order to assess the resistance of barley cultivars in a more accurate manner than symptomatic evaluation.

The aim of this study was to investigate differences in the *F. culmorum* response, determined as changes in *Tri* gene (*Tri4, Tri5, Tri6, Tri10*) transcription after infection of six barley genotypes differing in the pathogen resistance. The response of host plants was investigated as transcription of the barley glucosyltransferase gene (*HvUGT13248)* relative to the transformation of DON to D3G.

MATERIAL AND METHODS

Plant growing, inoculation and sampling. Experiments were carried out in greenhouse conditions at Kroměříž, Czech Republic. Six spring barley cultivars (Table 3) were grown in soil. At the beginning of anthesis (BBCH 61–65), trials were inoculated with *F. culmorum* by spraying [strain (W. G. Sm.) Sacc. KM16902; concentration of 0.5×10^6 conidia of *F. culmorum* in 1 ml of inoculum, spray dose of 200 l/ha]. Whole barley spikes were sampled individually before infection (control) and after 4, 7, 14, and 21 dpi at the same time of day in three independent replicates. The spikes were immediately frozen in liquid nitrogen and stored at –80°C.

DNA isolation, RNA isolation and purification. Frozen barley grains (0.1 g) were homogenised in liquid nitrogen, and genomic DNA was isolated using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Total RNA was isolated by phenol-chloroform extraction using TRIzol® Reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. The isolated RNA was purified using RNeasy Mini Kits and an RNase-Free DNase Set (Qiagen, Germany). The concentration and purity of the isolated nucleic acids were determined spectrophotometrically using a Nano-Photometer® (Implen, Munich, Germany). DNA and RNA integrity was verified using an electrophoretogram.

Preparation of cDNA. The isolated and purified RNA was reverse-transcribed using TaqMan® Reverse Transcription Reagents according to the manufacturer's instructions in a Verity® thermal cycler (Applied Biosystems, Forest City, USA).

RT qPCR and qPCR. The primers used to amplify the cDNAs of interest and references by RT qPCR are listed in Table 1. The reaction mixture contained 1 µl of cDNA corresponding to 20 ng of total RNA, 1× Power SYBR® Green PCR Master Mix (Applied Biosystems, USA), 200 nM of each primer, and nuclease-free water up to the final volume of 20 µl. Nuclease-free water was used as a negative control, and isolated and diluted (10 ng/µl) gDNA from *Fusarium culmorum* was used as a positive control. The amplification was performed in a StepOnePlus™ thermal cycler (Applied Biosystems, USA). The thermal profile of RT qPCR was 95°C (10 min), followed by 42 cycles of 95°C (30 s), the selected annealing temperature for each primer combination (Table 1) (30 s), and dissociation steps of 95°C (15 s), 60°C (1 min), and 95°C (15 s). Each of PCR amplifications was performed in triplicate for each of the three biological replicates. The specificity of the PCR products was checked by both dissociation curve and electrophoresis analysis. The PCR efficiency was determined using calibration curves made with 2× and 4× dilutions of a cDNA sample with the lowest quantification cycle (Cq) for a given primer pair. The coefficient of determination (R^2) for the standard curves ranged from 0.977 to 0.995.

The increase in pathogen DNA (pathogen amount) in the DNA isolated from the samples was measured by qPCR using a Taq Man MGB probe and two specific primers as described by Leišová *et al.* (2006) (Table 1). The amount of the pathogen was quantified using dilution series of a known amount of gDNA isolated from *F. culmorum* by qPCR as described above. A standard curve was constructed from the

https://doi.org/10.17221/21/2018-PPS

Table 1. The sequences of primer pairs and probe of target and reference genes used for RT-qPCR, including PCR efficiency value and annealing temperature

Primers/probe	Sequence (5'→3')	Product size (bp)	Reference	Efficiency	*T* (°C)
Fusarium culmorum					
Tri4-F	TATTGTTGGCTACCCCAAGG	95	Ponts *et al.* (2007)	2.00	57
Tri4-R	TGTCAGATGCGCCTTACAAA		Ponts *et al.* (2007)		
Tri5-F	GTGGGCACTTGTCAACGA	184 (cDNA)	Faltusová *et al.* (2015)	1.81	60
Tri5-R	ACCCAAACCGTTCATACGAC	244 (gDNA)	Faltusová *et al.* (2015)		
Tri6-F	CGGTGGATTCAACCAAGACT	180	Faltusová *et al.* (2015)	1.97	58
Tri6-R	AACTAGGGAATGGGCATTTG				
Tri10-F	GTGGGCTCGACAAGACATTT	105 (cDNA)	Faltusová *et al.* (2015)	1.95	58
Tri10-R	CCCTGCTTAGGTGCGAAGA	195 (gDNA)	Faltusová *et al.* (2015)		
UBC-PS.1	TCCCCTTACTCTGGCGGTGTC	104	Lysøe *et al.* (2006)	1.97	57
UBC-PS.2	TTGGGGTGGTAGATGCGTGTAGT		Lysøe *et al.* (2006)		
Fc 92s1-F	TTCACTAGATCGTCCGGCAG	92	Leišová *et al.* (2006)	1.92	60
Fc 92s1-R	GAGCCCTCCAAGCGAGAAG		Leišová *et al.* (2006)		
Fc 92s1 5´FAM 3´MGB probe	AAAGAAGTTGCAATGTTAGTG		Leišová *et al.* (2006)		
Hordeum vulgare					
GT-F	CATCGAGCCAAAGGAGGTAG	91	Faltusová *et al.* (2015)	1.90	57
GT-R	ACCATCGCCGAGGTAGTATG				
actin-F	ATCTCGCTGGTCGGGATCTCAC	101	Faltusová *et al.* (2015)	1.92	60
actin-R	GATGTCCCTTACAATTTCCCGCTC				
αtub-F	GCAACGCTTGCTGGGAGCT	95	Faltusová *et al.* (2015)	1.96	60
αtub-R	CGCATCGTGTGCAACCCCA				
βtub-F	AGGAGTACCCGGACCGCATGA	107	Faltusová *et al.* (2015)	2.00	60
βtub-R	AAGCTGGTGCACAGAGAGGGT				
EF1-F	ACTGCACCGTCATTGATGCCCCTG	101	Faltusová *et al.* (2015)	1.91	60
EF1-R	GGTGGAGTCAATGATGAGCACAGC				

T – annealing temperature

logarithmic value of fungal genomic DNA and qPCR data measured as Cq.

RT qPCR and qPCR data were collected and processed using Step One™ Software v 2.1 (Applied Biosystems, USA). The mathematical model of relative quantification published by Pfaffl (2001) was used to evaluate the target gene data. Data were related to those for the control samples collected prior to inoculation. The normalised transcription values were log-transformed. Statistical evaluation was by ANOVA and Fischer *LSD* test using Statistica (StatSoft, Inc.). The data sets were considered to be significantly different at $P < 0.05$.

Determination of DON and D3G contents. For the examination of DON and D3G contents 2 g of barley sample (pooled from three spikes) were weighed into a centrifugation cuvette and 20 ml of a 1 : 1 mixture of methanol and water (v/v) was then added. The samples were extracted using an ULTRA-TURRAX® dispenser (IKA Labortechnik, Staufen, Germany) for 3 min at 13 081 *g*. The samples were then centrifuged for 5 min at 13 081 *g*. A 1.5 ml aliquot of extract was filtered through a 0.2 µm PTFE microfilter (2 min at 3 270 *g*) and removed into an amber glass vial prior to injection for liquid chromatography-mass spectrometry. An ultra-high performance Accela 1250 U-HPLC liquid chromatography system with a Hypersil™ Gold analytical column (100 × 2.1 mm i.d. and 1.9-µm particle size; Thermo Fisher Scientific, USA) heated to 40°C and coupled to an Exactive™ high-resolution mass spectrometer (Thermo Fisher Scientific, USA) was used for quantifying DON and

https://doi.org/10.17221/21/2018-PPS

D3G. A gradient elution (A: 5 mM ammonium acetate, B: methanol) of the analysed mycotoxins was used for chromatographic separation. The method's run time was 10.5 minutes. Ionisation of the analysed mycotoxins was performed in negative mode using atmospheric pressure chemical ionisation (APCI). XCalibur™ software was used for data processing. The values for total DON produced (defined as TDP = DON [m/z = 296.32] + DON in D3G [m/z = 458.46]) and the proportion of DON converted to D3G (defined as PDC = DON in D3G/TDP) were calculated relative to molecular weight.

Table 2. Mean total deoxynivalenol (DON) content (TDP = DON + DON in D3G [DON-3-*O*-glucoside]) and proportion of DON converted to D3G (PDC = D3G/TPD) in different barley cultivars during 21-day infection by *Fusarium culmorum* (mean ± SE)

Cultivar	TDP (mg/kg FW)	PDC
Krasnodarskij 95	4.63 ± 1.21	0.23 ± 0.033
Amulet	0.70 ± 0.30	0.40 ± 0.030
Malz	1.00 ± 0.00	0.60 ± 0.000
Nordus	49.34 ± 18.33	0.17 ± 0.003
Diplom	16.45 ± 2.61	0.22 ± 0.000
Radegast	5.39 ± 1.24	0.17 ± 0.016

RESULTS

Comparison of pathogen amount, DON content and transcription Fusarium Tri genes in infected barley cultivars. Differences in pathogen contamination evaluated as DNA content of *F. culmorum* were found between barley cultivar samples. The highest content of the pathogen DNA was detected in cv. Nordus (at 7 dpi), followed by cv. Diplom (at 14 dpi) and in cvs Krasnodarskij95, Malz, and Radegast (at 21 dpi). The least infected cultivar was Amulet (Figure 1). Cultivars were similarly ranked regarding determination of their DON contents (Tables 2 and 3). A positive correlation was observed between *Fusarium* DNA and DON values (R^2 = 0.67) across the cultivars during infection. DON content in cv. Nordus was as much as 10 times higher than that in cvs Krasnodarskij95 and Radegast (Table 2). The correlation between *Fusarium* DNA and DON values was very high in the more severely infected cultivars Nordus and Diplom (R^2 = 1 and 0.998). The lowest DON content was determined in cvs Malz and Amulet. The largest amount of DON observed in cv. Nordus already at 7 dpi corresponded to an increase in the transcription of *Tri* genes from 4 dpi; the transcription peaked at 7 dpi in *Tri5* and *Tri4*. In cv. Diplom an increased transcription of *Tri5* and of *Tri4* at 7 dpi and 14 dpi was revealed, respectively. For both these genes, an increase was observed at 14 dpi in Krasnodarskij95. For *Tri4* an increase at 14 dpi was observed in Radegast (Figure 2).

In the case of regulatory genes, higher transcription of *Tri6* in cv. Krasnodarskij95 was observed and this gradually increased until 14 dpi with a subsequent decline. Cv. Radegast followed, showing a transient increase in the transcription of *Tri6* at 4 dpi. A highly significant transient increase was found in transcription of the *Tri10* gene in cv. Amulet between 3 and 7 dpi, while a much smaller transient increase was observed in cv. Malz at 7 dpi. A gradual slight increase in *Tri10* transcription was observed in cvs Nordus and Krasnodarskij95 (Figure 2).

Transcription of HvUGT13248 and conversion DON to D3G. Transcription of the UDP-glycosyl-

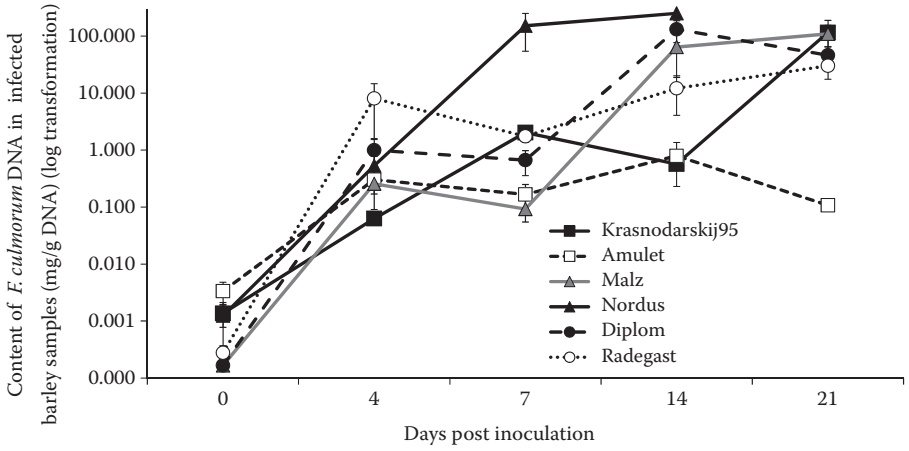

Figure 1. Amount of *Fusarium culmorum* pathogen DNA in DNA of infected barley samples (mg/g DNA)

Error bars represent ± SE (data of cv. Nordus were not measured at day 21)

https://doi.org/10.17221/21/2018-PPS

Table 3. Comparison of barley cultivars according to the resistance to FHB, amount of pathogen DNA, TDP (total DON (deoxynivalenol) content) and PDC (proportion of DON converted to D3G)

Resistance to FHB (Chrpová *et al.* 2011)	Amount of pathogen DNA (ranked from the highest value reached)	TPD (ranked from the highest value reached)	Average of PDC (ranked from low to high value)
Radegast (S)	Nordus	Nordus	Radegast
Diplom (MS)	Diplom	Diplom	Nordus
Malz (MS)	Krasnodarskij95	Krasnodarskij95	Diplom
Krasnodarskij95 (R-MR)	Malz	Radegast	Krasnodarskij95
Nordus (R-MR)	Radegast	Amulet	Amulet
Amulet (most resistant*)	Amulet	Malz	Malz

S – susceptibility; MS – moderate susceptibility; R – resistance; MR – moderate resistance; *Vančo *et al.* (2007)

transferase gene *HvUGT13248* corresponded to the amount of DON in the samples. A considerable increase in transcription of this gene was found in Nordus at 7 dpi (Figure 3). The total amount of DON (TDP) showed an exponential dependence upon PDC (the proportion of DON converted to D3G) (R^2 = 0.74 and Equation $y = 27867e^{-6,13x}$) across samples of all cultivars.

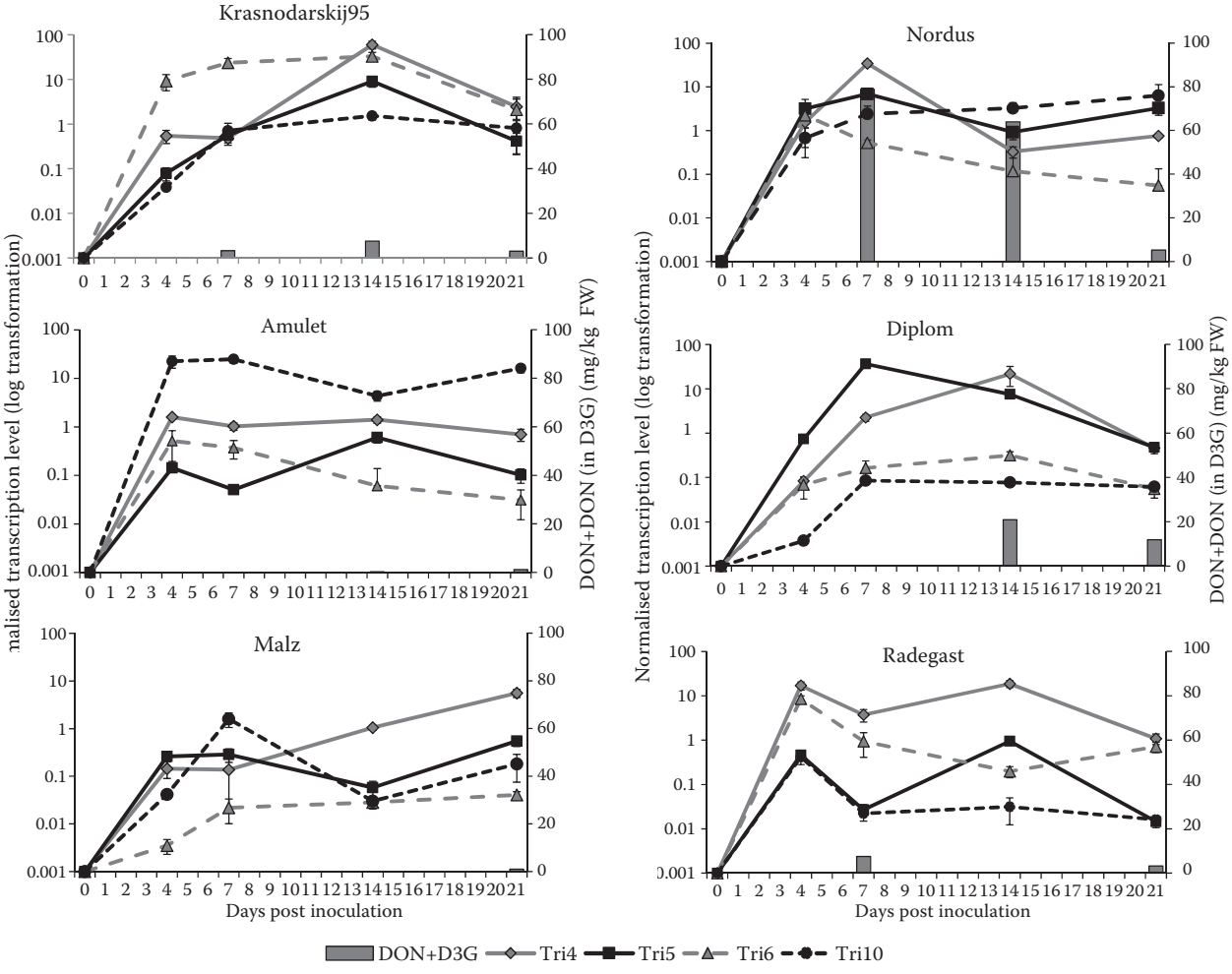

Figure 2. Transcription of *Fusarium culmorum Tri* genes compared to total DON (deoxynivalenol) content (calculated as DON+ DON in D3G [DON-3-*O*-glucoside]; mg/kg grains fresh weight – FW) in infected barley cultivars; error bars represent ± SE

https://doi.org/10.17221/21/2018-PPS

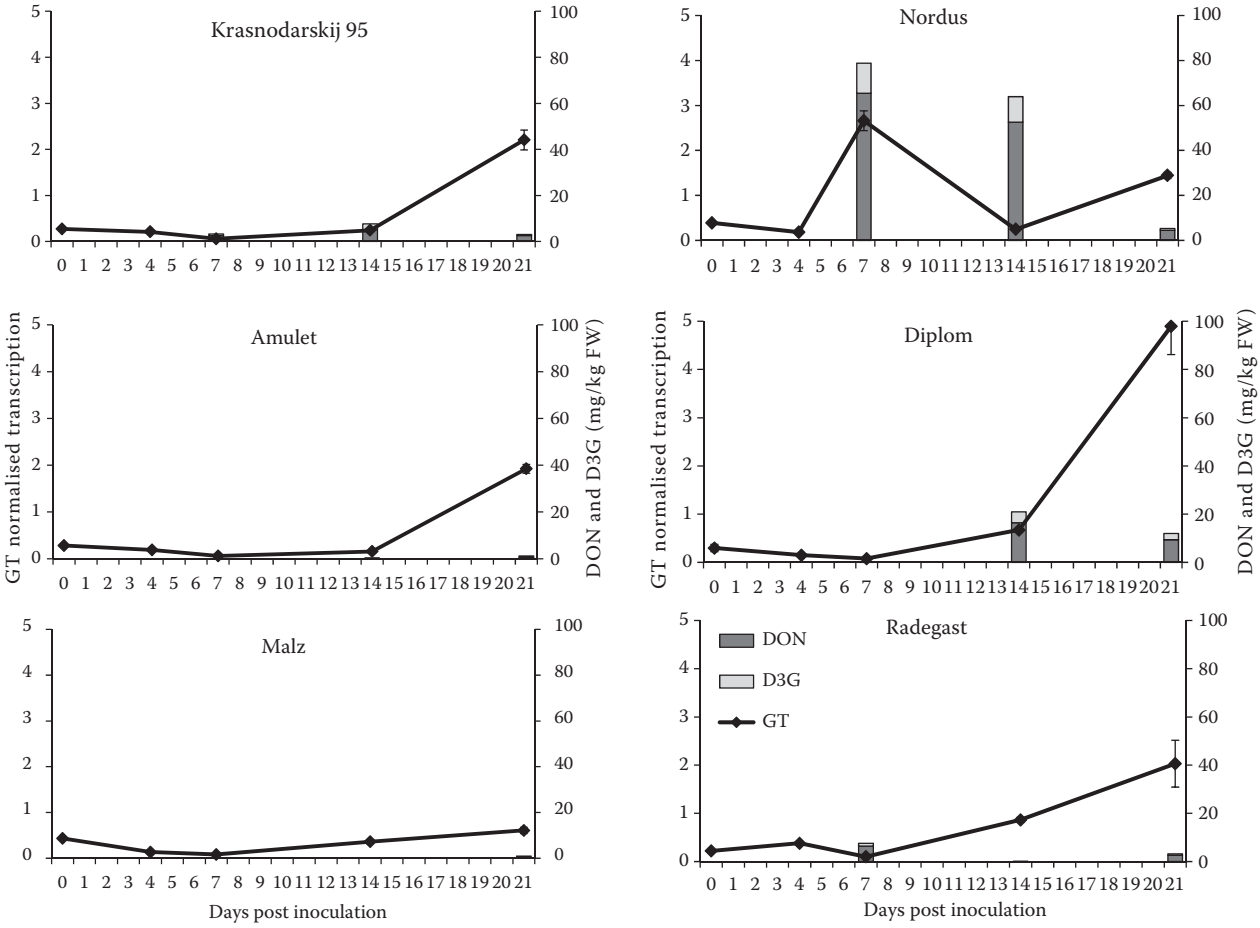

Figure 3. Transcription of GT (*HvUGT13248* gene) compared to DON (deoxynivalenol) and DON in D3G (DON-3-*O*-glucoside) content (mg/kg grain fresh weight – FW) in infected barley cultivars; error bars represent ± SE

With its only slight infection and low DON content, Amulet seems to be a resistant cultivar. Similarly, cv. Malz manifested the only gradual growth of the pathogen at 14 dpi even as its DON content was as low as Amulet's. Cv. Nordus, which has been characterised as resistant to FHB, exhibited the highest content of both pathogen DNA and mycotoxins. Cv. Malz declared as moderately sensitive had the lowest DON content (Table 3). PDC did not clearly reflect the cultivars' resistance evaluated as the amounts of the pathogen and DON contents in the samples (Table 3).

DISCUSSION

Since assessment using symptomatic evaluation of the disease in barley plants due to *Fusarium* is even more complicated than it is in wheat (Chrpová *et al.* 2011), we evaluated the infection as an amount of the pathogen by pathogen DNA content measurement in infected barley plants. Contamination of grains by DON reflects the amount of the pathogen DNA, and especially in more infected cultivars. Similarly, a positive correlation between *Fusarium* DNA and DON values was reported for barley (Demeke *et al.* 2010) and for wheat (Nicolaisen *et al.* 2009). In wheat, Bai *et al.* (2001) described significant correlation coefficients between FHB symptom ratings, seed quality traits, and DON levels. The fact that the content of the pathogen DNA correlates with the content of DON in more infected cultivars and that this correlation is not so evident in less affected cultivars in our study probably relates to the ability of resistant cultivars to somehow suppress the pathogen's synthesis of mycotoxins.

Transcription of *Fusarium Tri* genes *Tri4* and *Tri5* was greater in the most infected cultivars Nordus and Diplom, and this observation is consistent with the high DON content in these cultivars. In the case of regulatory genes, transcription differed between

https://doi.org/10.17221/21/2018-PPS

Tri6 and *Tri10*. While a transient increase of *Tri6* transcription was found in cultivars with moderate levels of DON (cvs Krasnodarskij95 and Radegast), transcription of *Tri 10* was temporarily increased in a cultivar with the highest DON levels (cv. Nordus). In the case of the second regulatory gene, *Tri10*, a higher transient increase in transcription was observed together with the lowest content of mycotoxins in cv. Amulet. This is consistent with our previous results from examining two barley cultivars, Chevron and Pedant. Transcription of *F. culmorum Tri* genes (especially *Tri10*) manifested a higher transient increase after infection of the resistant cultivar Chevron, but it did not lead to an increase of mycotoxins. The newly presented data support our earlier suggestion that a delayed and sharp rise in *Tri* gene transcription in the resistant barley cultivar indicates the pathogen reaction to enhance its infectivity by boosting DON production. The resistant cultivars, on the other hand, are able to inhibit this increase by some mechanism (Faltusová *et al.* 2015). As summarised by Merhej *et al.* (2012), many studies were aimed at the regulation of trichothecene biosynthesis in *F. graminearum*. Most of them showed an influence of environmental or extracellular factors on toxin production, but only a few studies elucidated the molecular regulation of *Tri* gene expression by highlighting the role of *Tri6* and *Tri10*. Although there have been studies addressing this issue (Hou *et al.* 2015; Jiang *et al.* 2016), more work is needed to elucidate the regulation of gene transcription by means of these regulatory factors. The conversion rate of DON to D3G did not always correlate with the level of resistance. This could be related to different mechanisms of resistance in the studied cultivars. Although cv. Nordus was proved to have the highest TDP, it also had the same low average value (0.17) for the DON conversion rate to D3G (PDC) as did cv. Radegast, which had a low amount of pathogen DNA. Very low content of pathogen DNA was measured also for cvs Amulet and Malz, but the conversion rates for these varieties were 0.40 and 0.60. Thus, no clear relationship between the degree of cultivar resistance and PDC was found. This is consistent with our recent study (Faltusová *et al.* 2015) and with that of Kumaraswamy *et al.* (2011), who concluded in agreement with Boutigny *et al.* (2009) that the D3G/DON ratio may not correctly identify the mechanisms of resistance inasmuch as the DON reduction can be due both to DON conversion to D3G and its reduced synthesis. Resistance based on a modification of trichothecenes is important in terms of the plant's own resistance. In tolerant genotypes, DON can be transformed to D3G, which is less toxic to plants but poses a potential risk to consumers in the event, for example, of its release from the conjugate during processing of contaminated kernels for food and in the digestive system of animals (Berthiller *et al.* 2011; Nagl *et al.* 2012). It is important to focus on breeding cultivars that allow pathogen infection or are somehow able to suppress the pathogen's synthesis of mycotoxins. In this study, cv. Amulet was identified as a cultivar more resistant to artificial infection by *F. culmorum* due to its low pathogen DNA content and low DON contamination. Determination of cv. Amulet as a resistant cultivar is consistent with the evaluation by Vančo *et al.* (2007). Comparing our results with the declared resistance of cultivars (Chrpová *et al.* 2011) shows no consistency. This is not consistent, however, with the work of Hajšlová *et al.* (2007), who observed the highest level of DON in cv. Amulet among 16 barley cultivars grown under natural conditions. Similar discrepancy was revealed between the evaluation of wheat cv. Ebi upon artificial *Fusarium* infection (Šíp *et al.* 2007) and in natural conditions evaluated by Hajšlová *et al.* (2007), who concluded that in fact there are many factors that influence DON levels in crops. These factors such as weather conditions around flowering, pathogen virulence and agricultural practices (crop rotation, soil cultivation, fertiliser, chemical treatment) were summarised by Edwards (2004).

This work and our previous study examining two barley cultivars differing in *Fusarium* resistance (Faltusová *et al.* 2015) suggest some ability of the host to delay transcription of the pathogen's *Tri* genes. We expect that further deeper examination of the *Tri* gene regulation transcription could help to find possibilities of decreasing mycotoxin production by *Fusarium*.

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