Efflux pumps as an additional source of resistance to trichothecenes in *Fusarium proliferatum* and *Fusarium oxysporum* isolates

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Abstract
Role of efflux-mediated toxin resistance to trichothecenes is known in trichothecene-producing species. However, the role of trichothecene efflux pump homologues in non-producing fusaria such as *F. oxysporum* and *F. proliferatum* was not investigated in detail. Analysis of the homologues of trichothecene efflux pump from multiple fungal species allowed us to uncover and catalogue functional gene copies of conserved structure. Putative Tri12 candidates in *Fusarium oxysporum* and *F. proliferatum* were characterised via expression profiling in response to different trigger compounds, providing supporting evidence for role of *Tri12* homologues in the resistance to trichothecenes. Our analysis of *Tri12* phylogeny also suggests that efflux-mediated trichothecene resistance is likely to predate the divergence of *Trichoderma* and *Fusarium* species. On the regulatory level, we posit that the increased tolerance of trichothecenes by *F. oxysporum* is possibly related to the decoupling of *Tri12* homologue expression from pH, due to the deletion of PACC/RIM101 transcription factor binding site in its promoter region.

Keywords Trichothecene efflux pump · *Fusarium oxysporum* · *Tri12* · Transcription factor binding site

Introduction
Mycotoxins are bioactive fungal secondary metabolites which are typically viewed mainly through the lens of their harmful properties to humans and livestock. Nevertheless, a large array of fungal pathogens spend most of their life cycle, in competition with prokaryotic and eukaryotic microbes (either persisting in the soil environment or competing for plant hosts). Thus, one can expect that many compounds affect fungal fitness through their effect on the microbe-microbe competition. In cases of some toxins, the divergence and spread of different toxigenic properties have been shown to predate the origins of major classes of filamentous fungi (e.g. Koczyk et al. 2015), with detoxification mechanisms likewise being ancient (e.g. Popiel et al. 2014; Perlin et al. 2014). In the different environments, the competitive impact of toxin biosynthesis can be positive, due to increased isolate virulence or toxin’s inhibitory effects on the growth of other strains (Losada et al. 2009).

In this context, there is evidence demonstrating that trichothecene biosynthesis has impact on fungus-fungus competition. For example, Ramakrishna et al. (1996) found that during competition between *F. sporotrichioides* (producer of T-2 toxin) and two other fungi: *A. flavus* and *Penicillium verrucosum* the growth of *F. sporotrichioides* was negatively affected but paradoxically the production of T-2 mycotoxin was stimulated. Similarly, Lutz et al. (2003) tested the impact of deoxynivalenol (DON) against a potent fungal antagonist *Trichoderma atroviride* and described decreased expression of genes coding chitinase in the presence of these toxins. In the studies of McLaughlin et al. (2009) and Bin-Umer et al. (2011), they observed the impact of trichothecenes on yeast cells. The results illustrated that mycotoxins can inhibit the mitochondrial membrane potential, translation and levels of reactive oxygen species in fungi, in a dose-dependent manner.

Trichothecene biosynthesis contributes to increased virulence of fungal strains, and further inquiries into other fungal
species show that the basic trichothecene scaffold is likely ancient at least within the context of multiple species within the Hypocreales order (Trichoderma arundinaceum and Trichoderma brevicompactum—Cardoza et al. 2011; Myrothecium roridum—Trapp et al. 1998; Stachybotrys sp.—Semeiks et al. 2014). While capacity for trichothecene biosynthesis is present in multiple members of the Hypocreales order, the Fusarium genus is perhaps the best characterised group (Kimura et al. 2007). Fusarium spp. are subdivided into related, but phylogenetically distinct complexes that likely diverged in cretaceous period (O’Donnell et al. 2013). These fungi widely differ in their preferences in regards to saprophytic and/or pathogenic lifestyles as well as biosynthetic capabilities (trichothecenes are mainly produced by members of incarnatum-equiseti and sambucinum complexes). Frequently, the diverged species find themselves in direct or indirect competition when their ecological niches overlap. The relationship between F. graminearum and F. verticilloides on cereals (including variability in trichothecene accumulation) (Picot et al. 2012; Dawidziuk et al. 2016) provides one example of this phenomenon.

Mirroring the ancient origins of trichothecene biosynthesis, effective trichothecene resistance mechanisms are known to be partially present in multiple producing and non-producing strains (Kimura et al. 2003; Tokai et al. 2005; Proctor et al. 2009; Menke et al. 2012). A good example is trichothecene efflux pump encoded by Tri12 gene in O-acetyltransferase Tri101 (and its divergent but functional Tri201 homologue). The Tri201 gene, in particular, was found to be present in both ancestrally divergent strains of Fusarium sp. from complexes other than sambucinum, e.g. the early diverging species F. decemcellulare, F. solani (Tokai et al. 2005) as well as in other species of fungi (Magnaporthe oryza—Tokai et al. 2005; Saccharomyces cerevisiae—Alexander et al. 2002).

While the trichothecene acetyltransferase homologues have been characterised in many species, the putative trichothecene efflux pump existence and functionality were not extensively investigated beyond the initial discovery of their functionality in the sambucinum complex (F. sporotrichioides—Alexander et al. 1999; F. graminearum—Wuchiyama et al. 2000). In producer species, the past comprehensive studies of Proctor and coworkers (Proctor et al. 2009; Cardoza et al. 2011) have shown that trichothecene efflux pump is frequently but not always present in the clade (e.g. incarnatum-equiseti complex fusaria). More recently, a brief survey of Tri12 domain encoding transporters was conducted by Perlin et al. (2014), where homologues of unconfirmed function were summarised across many saprobic, animal and plant pathogenic-species.

To establish whether trichothecene efflux is a likely retained trait in previously not investigated species (F. oxysporum, F. proliferatum), we studied presence and evolutionary history of divergent Tri12 homologues. The research was performed by combining phylogenetic analyses of available Ascomycota sequences with gene expression and bioassays. Through the phylogenetic analysis of multiple Tri12 homologues, we confirmed the notion that active resistance to trichothecene-like compounds is likely an ancient trait or one common enough to elicit a convergent evolution of multiple, distantly related resistance factors (acetyltransferase and active transport). In order to obtain supporting evidence for functionality of divergent Tri12 homologues in fujikuroi and oxysporum complex, we investigated the expression of F. proliferatum and F. oxysporum homologues in response to varying stimuli (including trichothecene presence).

Methods

Isolate collection and identification
From the culture collections available at the Institute of Plant Genetics, Polish Academy of Sciences, Poznan, Poland, we selected the following strains lacking capacity for trichothecene biosynthesis: eight F. oxysporum strains (10 L, 11 L, 19 L, 55 L, 57 L, 94 L, 115 L, 131 L) with no recorded toxigenic potential (fumonisins, trichothecenes, zearalenone) and ten F. proliferatum strains (1 L, 3 L, 7 L, 21 L, 36 L, 58 L, 59 L, 66 L, 81 L, 99 L) known to produce fumonisins. Fungal strain annotation was conducted as per the protocol described in Dawidziuk et al. (2014) on basis of both morphological and molecular data: F. oxysporum strains 10 L—GenBank Accession number MN018756, 11 L-MN018757, 19 L-KF889103, 55 L-KF889104, 57 L-KF889105, 94 L-MN018758, 115 L-KF889099, 131 L-KF889101 and F. proliferatum strains 1 L-KF889131, 3 L-KF889134, 7 L-KF889137, 21 L-KF889122, 36 L-MN018759, 58 L-KF889136, 59 L-KF889103, 66 L-KF889125, 81 L-MN018760, 99 L-KF889127).

Bioassays with multiple compounds
For the purpose of the bioassay experiment, the concentration of deoxynivalenol was set to 8 mg/L as the lowest dose inhibiting fungal growth. The lower concentrations of toxin (1 mg/L, 2 mg/L and 5 mg/L) did not significantly influence growth of the isolates and the higher doses (10 mg/L) inhibited growth of all fungal cultures.

The additional bioassay experiments were carried out to eliminate the impact of environmental factors on the growth of the tested cultures: MgCl2, KCl, ferulic acid, fungicide-Alert 350 SC (flusilazole) ground wheat seedlings, glucose, sucrose, coumaric acid, H2O2, caffeine, F. verticilloides (fumonisin producer). The concentration of additional chemical compounds was set to the same value as the concentration of DON. In the case of additional biological compounds, the 5 g of ground wheat was added to 250 mL of PDA medium.
and *F. oxysporum*/*F. proliferatum* bioassays with *F. verticillioides* was tested in the dual cultures (Gromadzka et al. 2009). The response was observed on PDA medium amended in simulated day (16 h)/night (8 h) conditions at 25 °C. The *F. oxysporum* assay was performed in three biological and ten technical replicates and the *F. proliferatum* assay was performed in three biological and ten technical replicates. Biological replicates were performed separately in the phytotron strictly controlling temperature, humidity and simulated day/night conditions.

The surface area of the fungal colonies was calculated by approximating the mycelium’s area to an ellipse by measuring both the length and width of the mycelium 4 days after toxin exposition (Dawidziuk et al. 2016).

**Isolation of DNA and sequencing**

Mycelium used for DNA extraction was obtained by inoculating Czapek-Dox broth (Sigma Aldrich, St. Louis, Missouri, USA) with yeast extract (Oxoid, Waltham, Massachusetts, USA) and streptomycin sulphate (50 mg/L, AppliChem, Darmstadt, Germany) and after incubation at 25 °C on a rotary shaker (120 rpm). Mycelium was collected on filter paper in a Büchner funnel, washed with sterile water, frozen at −20 °C and freeze-dried. Total DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The quality of DNA was estimated by a NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific, Wilmington, USA) and a Experion Automated Electrophoresis System (Bio-Rad, Hercules, CA). The protocols for primer design, PCR and sequencing conditions have been previously described by Popiel et al. (2014) and sequences of the primers are listed in Table 1.

**Annotation of Tri12 homologues**

The putative *Tri12* homologues were gathered using a variant of the approach used in our previous work (Koczyk et al. 2015). Briefly, first a wide set of homologues was compiled through BLASTP searches against the combined NCBI/nr database (26/10/2015) and a local copy of Ensembl/Fungi release 28. The combined database was made non-redundant by clustering at 97% protein sequence identity with CD-HIT (Fu et al. 2012), representative sequences were inspected and kept on a per-species bases. The sequences of *Tri12* from *F. sporotrichioides* and *F. graminearum* were used as queries.

To obtain the final set of *Tri12* homologues, we performed unsupervised clustering with model transporters of known specificity. For this, the preliminary subset of candidates was combined with all available 2.A.1.3 (DHA14 antiporter family) homologues from Transporter Classification Data Base (Saier et al. 2016). The clustering was conducted in CLANS (Frickeny and Lupas 2004) based on exhaustive all against all BLASTP comparisons with an expect value threshold of E-10. The stability of cluster containing *Tri12* homologues was validated at more restrictive similarity thresholds (cluster membership was tested up to 1e-80 expect value threshold). The final set, used for alignment and phylogeny reconstruction, numbered a total of 33 sequences, after also including the two sequences corresponding to protein sequence consensus of, respectively, *F. proliferatum* and *F. oxysporum* *Tri12* sequences obtained from the collection isolates.

We opted for the above iterative approach, as the simple selection, e.g. based on the conserved Pfam domain (TR112) fingerprint, would result in a large set of poorly alignable distant homologues. Multiple sequences with *Tri12* similarities are, upon inspection, DHA14 transporters of completely different specificity (such as STR1—the siderophore iron transporter from *Schizosaccharomyces pombe*, Pelletier et al. 2003) or present characteristic features of multidrug transporters (e.g. SGE1—violet/multidrug resistance protein from *Saccharomyces cerevisiae*, Ehrenhofer-Murray et al. 1998).

Where referenced, annotation of putative transcription factor binding sites was carried out in JASPAR (Mathelier et al. 2015). Putative transmembrane elements were annotated with CCTOP (Dobson et al. 2015) and TOPCONS (Tsirigos et al. 2015).

**Sequence alignment and phylogeny reconstruction**

The selected protein sequences were aligned with MAFFT-LINSI v 7.221 (Katoh and Standley 2013). For phylogenetic analysis, the multiple alignment was filtered with TCOFFEE/TCS module (Chang et al. 2014) using the transitive consistency score of 2 as the threshold (as recommended by the authors). The nucleotide sequences from the examined *F. oxysporum* (8 sequences) and *F. proliferatum* (10 sequences) isolates were aligned with MAFFT-LINSI and manually inspected for alignment correctness (referring to the earlier protein sequence alignment). For use in phylogeny reconstructions, a *F. sporotrichioides* reference *Tri12* sequence as well as additional model *F. oxysporum* (4 sequences) and *F. fujikuroi* (1 sequence) sequences were added to this alignment.

Both nucleotide and protein, maximum likelihood phylogeny reconstructions were carried out with IQTREE v 1.3.6 (Nguyen et al. 2015), using built-in model selection and ultra-fast bootstrap (Minh et al. 2013) procedure. In case of nucleotide sequences, this analysis was carried out in partitioned mode, with separate models for each exon and intron (auto-selected by IQTREE).

The full alignments of both nucleotide and protein sequences, used for phylogenetic reconstructions, are included in the *Supplementary Materials* to this article. The alignments were visualised in CLC Genomics Workbench v 8.5.1 (Qiagen) and the phylogenetic trees were drawn with
MEGA (nucleotide sequence-based tree, Tamura et al. 2013) and ETE2 (protein tree, Huerta-Cepas et al. 2010). The relevant gene structures were annotated and visualised with WebScipio (Hatje et al. 2011).

Expression profiling

Mycelium was collected from the medium and each sample was weighed on a laboratory scale (due to rapid RNA degradation, wet weight was analysed) (Sartorius AG, Göttingen, Germany). Total RNA from chosen, representative isolates was purified using an RNasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturers’ protocol with the additional DNase digestion step. The quality of total RNA was estimated by Nanodrop (Thermo Scientific, Wilmington, DE) and via Bioanalyzer (Bio-Rad, Hercules, CA). RNA dissolved in DEPC water was stored at −80 °C.

qRT-PCR primers were designed on the basis of previously sequenced gene fragments using Primer 3 and their properties were tested using OligoCalc.

Real-time RT-PCR was used to amplify Tri12 homologues (trichothecene efflux pump) and Tri201 (homologue of trichothecene 3-O-acetyltransferase from F. graminearum) in F. oxysporum and F. proliferatum strains (Desjardins and Proctor 2007; Lee et al. 2011), and as a reference, we used housekeeping genes Tub2 (β-tubulin), UBC (ubiquitin) and TEF1-α (translation elongation factor) from each RNA sample of the fungal strains.

Real-time RT-PCR reactions were performed using an CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA). Analyses were conducted using iTaqq One Step SYBR Green RT-qPCR Kit (Bio-Rad, Hercules, California, USA). The total reaction volume was 25 μL: 12.5 μL iTaqq One Step SYBR Green RT-qPCR mix, 1 μL RNA (≤35 ng), 0.5 μL each primer (10 μM), 0.125 μL reverse transcriptase and 5.125 μL nuclease free water. The reaction was carried out using the following protocol: initial

| Gene targeted | Primer name | Primer sequence (5' to 3') | Protocol         |
|---------------|-------------|-----------------------------|------------------|
| Trichothecene efflux pump (Tri12) | preTRI12_F1 | ACACGACGACATCACCTGCA       | Amplification and sequencing |
|                | preTRI12_R1 | GCGCGGAGTGGAAGAGGCTA       |                  |
|                | preTRI12_F2 | ACACGACGACATCACCTGCA       |                  |
|                | preTRI12_R2 | GCGCGGAGTGGAAGAGGCTA       |                  |
|                | preTRI12_F3 | ACACGACGACATCACCTGCA       |                  |
|                | preTRI12_R3 | GCGCGGAGTGGAAGAGGCTA       |                  |
|                | preTRI12_F4 | ACACGACGACATCACCTGCA       |                  |
|                | preTRI12_R4 | GCGCGGAGTGGAAGAGGCTA       |                  |
|                | TRI12_F1   | GAGAATCATCATATACTTCCAA     |                  |
|                | TRI12_R1   | GAGAATCATCATATACTTCCAA     |                  |
|                | TRI12_F2   | GAGAATCATCATATACTTCCAA     |                  |
|                | TRI12_R2   | GAGAATCATCATATACTTCCAA     |                  |
|                | TRI12_F3   | GAGAATCATCATATACTTCCAA     |                  |
|                | TRI12_R3   | GAGAATCATCATATACTTCCAA     |                  |
|                | TRI12_F4   | GAGAATCATCATATACTTCCAA     |                  |
| Homologue of trichothecene 3-O-acetyltransferase (Tri201) | rta_tr1201_F1 | TACATCTCCGGCAATCTA          | Gene expression |
|                | rta_tr1201_R1 | GTGGTGCTTCCAAGAAGATGTA     |                  |
| Translation elongation factor 1-α (TEF1A) | rta_tef1a_F1 | GCCAAAGCAAGGCTGGGATT       | Gene expression |
|                | rta_tef1a_R1 | GCCAAAGCAAGGCTGGGATT       |                  |
| Ubiquitin (UBC) | rt_UBC_F1   | GGCACAGAAATTGAGTCGAGGTT   | Gene expression (reference gene) |
|                | rt_UBC_R1   | GGCACAGAAATTGAGTCGAGGTT   |                  |
| β-tubulin (TUB2) | BtubF      | GCGGACGACGAGATAATGTGTT    | Gene expression (reference gene) |
|                | BtubR       | GCGGACGACGAGATAATGTGTT    |                  |
denaturation 94 °C for 2 min, followed by 40 cycles at 94 °C for 15 s, 59 °C for 1 min. In the experiment, we used three biological and two technical replicates together with a template-free negative control in each analysis of both target and control genes. The melting curve analysis (from 70 to 95 °C) confirmed primer pair specificity. As a control, we used mycelium samples cultivated on medium without the addition of toxins. Relative quantification of gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Bio-Rad, Hercules, CA). Data from samples treated with mycotoxin were normalised to β-tubulin, ubiquitin, 1-α translation elongation factor genes as internal controls (Real-Time PCR Application Guide, Bio-Rad, Hercules CA).

### Statistical analyses

Statistical analyses of growth patterns (relative area on the Petri dish covered by fungus) comprised analyses of variance (ANOVA) and post hoc means comparisons (Tukey-Kramer honestly significant difference [HSD]; $p \leq 0.05$) were performed with the Statistica 9.0 software package (Stat Soft, USA). The differences in gene expression between untreated and treated samples were analysed with Wilcoxon signed-rank non-parametric test ($p \leq 0.05$) with use of one-tailed hypothesis. The test was performed on the delta Ct values from the second day after the exposition to the indicated compound(s).

### Results

#### Growth patterns of strains treated with deoxynivalenol

Among all tested isolates of *F. oxysporum* and *F. proliferatum*, the response to deoxynivalenol in concentration of 8 mg/L was weak but still significant. Greater differences were noted between species. Growth of fumonisin producing *F. proliferatum* strains was inhibited by an average of 13% and of non-producing *F. oxysporum* by 6%. Importantly, in case of *F. oxysporum* isolates, one (10 L) was significantly different (more similar to *F. proliferatum* strains) and its growth was reduced by 10% (Table 2). Addition of lower doses of mycotoxin did not result in significant growth inhibition and higher concentration of deoxynivalenol in the medium suppressed the growth of all tested strains without any exceptions.

Growth of selected isolates in the control environment after addition of MgCl$_2$, KCl, glucose, sucrose, *F. verticillioides* did not show significant changes ($p \leq 0.05$) while the rest of the additives (ferulic acid, ground wheat seedlings, coumaric acid, H$_2$O$_2$, caffeine) caused significant reduction of the mycelium growth rate (Table 3).

#### Transcriptional response of *Tri12* homologues in *F. oxysporum* and *F. proliferatum*

The expression of *Tri12* homologue genes was analysed in *F. oxysporum* and *F. proliferatum* strains after deoxynivalenol treatment. As a reference, *Tub2* (β-tubulin), *UBC* (ubiquitin) and *TEF1-α* (translation factor) genes were used. In the presence of mycotoxin, *Tri12* gene in *F. oxysporum* strain (11 L) has shown significantly ($P \leq 0.05$) increased transcriptional activity (13.45-fold change–96 h after toxin exposition). Weakest induction was observed in deoxynivalenol treated *F. proliferatum* isolate, in which the relative normalised expression of *Tri12* homologue was 2.92-fold increased. Interestingly, *F. oxysporum* strain (10 L) showing similar growth patterns to the *F. proliferatum* isolates also indicated analogous *Tri12* expression (3.06-fold–96 h after toxin exposition) (Table 2). Specificity of the transcription induction of *Tri12h* homologue genes in *F. oxysporum* (trichothecene transporter) was confirmed by the profiling of *Tri12* gene expression in the presence of the multiple chemical compounds some of which can be potentially transported by the broad specific efflux pumps (Table 3). Only addition of deoxynivalenol significantly increased expression of *Tri12* gene (13.45-fold). In the case of the rest used substances, addition of potentially harmful ferulic acid, coumaric acid, fusiclode (Alert 375 SC), caffeine, H$_2$O$_2$ caused significant ($P < 0.05$) decrease of transcript level. Addition of sugars (glucose, sucrose) microelements (MgCl$_2$, KCl) and potential host tissues (wheat leaves and roots) did not influence gene expression.

#### Transcriptional response of *Tri201* homologues in *F. oxysporum* and *F. proliferatum*

To analyse general response of *F. oxysporum* and *F. proliferatum* strains to the presence of deoxynivalenol in the environment, expression of *Tri201* gene was tested (*Tri201* is a homologue of *Tri101*gene—responsible for detoxification by 3-O-acetylation of the trichothecene skeleton in the biosynthetic pathway in *F. graminearum*). In the presence of mycotoxin, *Tri201* gene in *F. oxysporum* strains has shown significantly ($P \leq 0.05$) increased transcriptional activity (13.07-fold–96 h after toxin exposition). No induction was observed in deoxynivalenol-treated *F. proliferatum* isolates.

#### Sequence and phylogeny of *Tri12* homologues

The protein sequences of *Tri12* cluster members were clearly alignable with conserved transmembrane regions and (to a degree—Figs. 1 and 2) conserved splice junction positions in relation to the multiple sequence alignments.

The subsequent phylogenetic reconstruction of evolutionary relationships between *Tri12* homologues (Fig. 2)
has confirmed the distant relationship between canonical Tri12 genes present in sambucinum complex fusaria and more distant homologues (referred to as Tri12b) found in oxysporum and fujikuroi complexes. Majority of bipartitions were strongly supported (> 70% support) in ultrafast bootstrap analysis.

### Table 2
Reduction of F. oxysporum and F. proliferatum relative surface area treated with 8 mg/L of deoxynivalenol. Surface area is calculated relative to mean surface area of control samples at the fourth day of measurements.

| Strain | Species     | Area of the colony in the comparison to the control (1%) | F statistic | p value | Tukey HSD Q statistic | Tukey HSD inference |
|--------|-------------|----------------------------------------------------------|-------------|---------|------------------------|---------------------|
| 10 L   | F. oxysporum| 89                                                       | 2.443       | 0.0202  | 5.1073                 | *p < 0.05           |
| 11 L   |             | 94                                                       | 0.4509      | insignificant |                        |                     |
| 19 L   |             | 95                                                       | 0.9205      | insignificant |                        |                     |
| 55 L   |             | 94                                                       | 2.1203      | insignificant |                        |                     |
| 57 L   |             | 95                                                       | 0.263       | insignificant |                        |                     |
| 94 L   |             | 94                                                       | 0.9195      | insignificant |                        |                     |
| 115 L  |             | 95                                                       | 0.9644      | insignificant |                        |                     |
| 131 L  |             | 94                                                       | 0.5918      | insignificant |                        |                     |
| 3 L    | F. proliferatum| 85                                                      | 29.1237     | 0.0202  | 15.2175                 | *p < 0.05           |
| 7 L    |             | 84                                                       | 14.5213     | *p < 0.05 |                        |                     |
| 21 L   |             | 87                                                       | 19.6747     | *p < 0.05 |                        |                     |
| 36 L   |             | 88                                                       | 11.4717     | *p < 0.05 |                        |                     |
| 58 L   |             | 87                                                       | 18.3228     | *p < 0.05 |                        |                     |
| 59 L   |             | 86                                                       | 15.3910     | *p < 0.05 |                        |                     |
| 66 L   |             | 85                                                       | 15.9318     | *p < 0.05 |                        |                     |
| 81 L   |             | 87                                                       | 15.1864     | *p < 0.05 |                        |                     |
| 99 L   |             | 88                                                       | 14.0857     | *p < 0.05 |                        |                     |
| 1 L    |             | 89                                                       | 15.2548     | *p < 0.05 |                        |                     |

### Table 3
Expression (N-fold) of Tri12 homologue and reduction of mycelium area in F. oxysporum (mean of all tested isolates except 10 L) and F. proliferatum (mean of all tested isolates except 10 L) treated with different chemical and biological substances.

| Additive          | Gene expression (N-fold) | Growth rate (mm) |
|-------------------|--------------------------|------------------|
|                   | F. oxysporum | F. proliferatum | F. oxysporum (10 L) | F. oxysporum | F. proliferatum | F. oxysporum (10 L) |
| Control           | 1.22         | 1.13           | 1.18                 | 33.58       | 48.3           | 33.58 |
| Deoxynivalenol    | 13.45*       | 2.92*          | 3.06*                | 31.58**     | 41.94**        | 30.18** |
| E. verticillioides| 0.94         | 0.95           | 0.98                 | 32.26       | 48.65          | 34.35 |
| MgCl2             | 0.97         | 0.98           | 0.44                 | 33.51       | 48.81          | 34.35 |
| KCl               | 1.45         | 1.28           | 1.40                 | 34.53       | 49.25          | 34.51 |
| Ferulic acid      | 0.42         | 0.39           | 0.41                 | 30.83**     | 42.84**        | 30.82** |
| Wheat             | 0.69         | 0.74           | 0.67                 | 31.57**     | 45.4           | 29.89** |
| Glucose           | 0.73         | 0.69           | 0.75                 | 36.6        | 52.65          | 34.02 |
| Alert 375 SC (flusilazole) | 0.46 | 0.52 | 0.47 | 14.10** | 24.63** | 15.11** |
| sucrose           | 0.89         | 0.85           | 0.91                 | 36.6        | 52.65          | 36.13 |
| Coumaric acid     | 0.29         | 0.35           | 0.31                 | 29.81**     | 38.50**        | 29.33** |
| H2O2              | 0.31         | 0.29           | 0.33                 | 30.74**     | 44.01**        | 30.60** |
| Caffeine          | 0.37         | 0.35           | 0.36                 | 29.48       | 38.01**        | 29.32** |

*Result is significant at p ≤ 0.05 (Wilcoxon signed-rank non-parametric test with use of one-tailed hypothesis)

**Result is significant at p ≤ 0.05 (analyses of variance (ANOVA) and post hoc means comparisons—Tukey-Kramer honestly significant difference
However, the attempts to root the resulting trees with even more distant homologues from DHA14 subset of MFS1 transporters have led to inconsistent results. This is likely due to ‘twilight zone’ levels of sequence similarity (around 20% protein sequence identity; BLAST expect values < 1e-20) in comparison to the considered outgroups (the sequences from 2.A.1.3 level of TCDB classification of transporters, e.g. Mfs1 from T. harzianum, Vba5p from Saccharomyces cerevisiae).

Thus, we have opted for midpoint rooting in our reconstruction of the Tri12 ancestry (Hess and De Moraes Russo 2007). Nucleotide sequence comparisons between the F. oxysporum and F. proliferatum isolates have shown the monophyleticity as members of their respective species complexes (Fig. 3—maximum likelihood tree). The sequence alignments have also uncovered a 25-26 bp indel differentiating between F. oxysporum and F. fujikuroi/proliferatum promotor regions (Fig. 4).
this difference is possibly tied to the observed divergence in expressional patterns (see the following section for details). Interestingly, in *F. oxysporum* strain (10 L) showing similar growth patterns to the *F. proliferatum* isolates, sequence alignments have also uncovered a similar indel (Fig. 4). All *Tri12* homologue sequences are available in NCBI database (KX273324, KX273325, KX273326, KX273327, KX273328, KX273329, KX273330, KX273331, KX273332, KX273333, KX273334, KX273335, KX273336, KX273337, KX273338, KX273339, KX273340, KX273341).
Discussion

Since the initial discovery and experimental characterisation of Tri12 efflux pomp (Alexander et al. 1999), subsequent inquiries have established its role in self-protection and virulence of trichothecene-producing strains (Menke et al. 2012). However, while the transformative detoxification mechanism in the form of Tri101 O-3-acetyltransferase was found to be crucial in trichothecene resistance in multiple producing and non-producing species, the deletion experiments pointed to less significant role of the active efflux (Kimura et al. 2007; Khatibi et al. 2011). The presence and possible involvement of Tri12 homologues in non-producing species were largely left uninvestigated.

Our phylogeny reconstruction results (see Fig. 2) support early divergence of canonical Tri12 homologues in the Fusarium genus (present mostly in the sambucinum complex of the genus, as well as Trichoderma arundinaceum and a single, early diverging F. oxysporum isolate (10 L). The ordering and included sequences from model genomes are same as on the Fig. 3
Trichothecenes, which are produced by the Fusarium genus, are a group of mycotoxins known for their potent effects on plant growth. They are synthesized through a complex biosynthetic pathway, which involves the action of specific enzymes and transporters. The presence of these toxins can cause significant economic losses in agriculture, as well as health issues in humans and animals.

The authors of this study aimed to elucidate the molecular mechanisms underlying the resistance of Fusarium oxysporum isolates to trichothecenes. They focused on the comparison of the transporter gene expression in resistant and non-resistant isolates. The study highlighted the importance of the transporter gene, TaTri12, in the self-resistance of producers and the possibility of its role in the transport of trichothecene-related toxins.

The authors observed that the variation in resistance to trichothecenes could be attributed to differences in the TR12 promoter region and overall transcriptional response. This is consistent with previous studies indicating that the TR12 promoter region is important for the expression of the trichothecene biosynthetic genes.

In conclusion, the study provides valuable insights into the molecular mechanisms of trichothecene resistance in Fusarium oxysporum. Understanding these mechanisms is crucial for developing strategies to control these mycotoxins in crop production.

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Compliance with ethical standards
Conflict of interest
Delphina Popiel declares that she has no conflict of interest.
Adam Dawidziuk declares that he has no conflict of interest.
Grzegorz Koczyk declares that he has no conflict of interest.

Ethical approval
This article does not contain any studies with human participants or animals performed by any of the authors.

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