A mycotoxin transporter (4D) from a library of deoxynivalenol-tolerant microorganisms

Celia Jimenez-Sanchez a, Nina Wilson a, Nicole McMaster a, Dash Gantulga a, Benjamin G. Freedman b, Ryan Senger b, David G. Schmale III a, b

a School of Plant and Environmental Sciences, Virginia Tech, Blacksburg, VA, 24061-0390, USA
b Department of Biological Systems Engineering, Virginia Tech, Blacksburg, VA, 24061, USA

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ABSTRACT

New strategies are needed to mitigate the mycotoxin deoxynivalenol (DON) in feed and food products. Microbial DNA fragments were generated from a library of DON-tolerant microorganisms. These fragments were screened in DON-sensitive yeast strains for their ability to modify or transport DON. Fragments were cloned into a PCR8/TOPO vector, and recombined into the yeast vector, pYES-DEST52. Resulting yeast transformants were screened in the presence of 100 ppm DON. Transformants that were able to grow in the presence of DON were plated on a selective medium, and the cloned microbial DNA fragments were sequenced. BLAST queries of one microbial DNA fragment (4D) showed a high degree of similarity to an ABC transporter. A series of screening and inhibition assays were conducted with a transport inhibitor (propanol), to test the hypothesis that 4D is a mycotoxin transporter. DON concentrations did not change for yeast transformants expressing 4D. The ability of yeast transformants expressing 4D to transport DON was inhibited by the addition of propanol. Moreover, yeast transformants expressing a known efflux pump (PDR5) showed similar trends in propanol transport inhibition compared to 4D. Future work should consider mycotoxin transporters such as 4D to the development of transgenic plants to limit DON accumulation in seeds.

1. Introduction

Food and feed products can be contaminated with toxic metabolites produced by fungi called mycotoxins. Deoxynivalenol (DON) is a mycotoxin produced by the fungus Fusarium graminearum. DON is a contaminant of staple crops such as wheat, barley, and maize (Binder et al., 2007). DON causes deleterious effects when consumed by domestic animals including feed refusal, vomiting, reduced weight, and even death (Rotter et al., 1996). The mode of action for DON occurs via interfering with initiation and elongation of protein synthesis (Pestka, 2010; Ueno et al., 1973). Traditional breeding and fungicide applications have been the main means to mitigate DON in crops (Khatibi et al., 2011). However, new strategies are needed to mitigate DON.

The epoxide ring present on C-12, 13 of DON is critical for toxicity (Karlovsky, 2011). Since the 1990s, there have been reports of microorganisms able to either modify or detoxify DON (McCormick, 2013). Microorganisms able to oxidize DON to the less toxic metabolite 3-keto-4-deoxynivalenol (3-keto-DON) have been reported in Shima et al. (1997) (Shima et al., 1997) and Völkl et al. (2004) (Völkl et al., 2004). Iikunaga et al. (2011) (Iikunaga et al., 2011) reported a species in the genus Nocardioides that was able to epimerize DON to 3-epimer-deoxynivalenol (3-epi-DON). More recently, a mixed culture of bacteria discovered by He et al. (2016) (He et al., 2016) was reported to convert DON to the less toxic de-epoxy-DON (DED). The modification of DON by acetylation (Kimura et al., 1998a, 1998b), epoxidation (Binder et al., 2007; Fuchs et al., 2002, 2000), or glucosylation (Poppenberger et al., 2003) produced secondary metabolites of DON that are reduced in their toxicity. Other microorganisms, including Aspergillus tubingensis (He et al., 2008), Nocardioides sp. (Iikunaga et al., 2011), and Devosia sp. (Sato et al., 2012) have been reported to be effective in the microbial degradation of DON. A recent study described the ability of the bacterium Devosia insulae to transform DON (Wang et al., 2019).

An alternative strategy for mitigating DON is to increase the transport of the mycotoxin out of cells. DON has been found to be transported by two of the main known groups of efflux pumps, ATP-binding cassette (ABC) transporters and major facilitator superfamily (MSF) transporters.
The ABC transporter Pdr5p was shown to be an exporter of DON and 15-
ADON in Saccharomyces cerevisiae (Gunter et al., 2016; Mitterbauer and Adam, 2002; Suzuki and Iwahashi, 2012). The MFS transporter TRU12 was shown to be trichothecene efflux pump (Alexander et al., 1999).

Wilson et al. (2017) showed that microorganisms isolated from environmental samples could modify DON to less toxic products. Here, we report the identity of a unique DON transporter (4D) from a library of microbial DNA fragments collected by Wilson et al. (2017) (Wilson et al., 2017). The microbial DNA fragments were generated using a technique known as oligonucleotide primed polymerase chain reaction (DOP-PCR); (2) screen the library of microbial DNA fragments from DON-tolerant microorganisms described in Wilson et al. (2017) using degenerate oligonucleotide primed polymerase chain reaction (DOP-PCR); (3) identify fragments capable of modifying or transporting DON; and (4) conduct experiments to demonstrate the role of a putative transporter (4D) to transport DON out of yeast cells.

2. Materials and methods

2.1. Generation of microbial library fragments

Microbial library fragments were generated from one pure culture of bacteria (Pure Culture 1) and three mixed cultures (Mixed Culture 1, Mixed Culture 2, and Mixed Culture 3) from Wilson et al. (2017). These samples came from a series of plant and soil collections, and were processed following the schematic shown in Fig. 1. A 50 μl sample from the glycerol stock was taken and cultured in 2 ml of Reasoner’s 2A (R2A, VWR, Radnor, PA) broth. DNA was extracted from the four cultures using a DNeasy PowerSoil Kit (Mo Bio Laboratories, Carlsbad, CA). Primers from the M set (M1, M2, M4 and M5) and Rand3 (Freedman, 2014) were used to generate library fragments via DOP-PCR from Pure Culture 1, Mixed Culture 1, Mixed Culture 2, and Mixed Culture 3 from Wilson et al. (2017). The DOP-PCR parameters involved 10 cycles of low stringency amplification with a 5 min denaturation at 95 °C followed by 10 cycles of: 94 °C for 1 min, 30 °C for 2 min, ramp to 68 °C for 3 min, and amplification at 68 °C for 8 min. Following this program, a high stringency program of 25 cycles was implemented. Each cycle consisted of: 94 °C for 1 min, 55 °C for 2 min, and 68 °C for 8 min, with 5 s added for each cycle. The DOP-PCR products were then held at 68 °C for 7 min. The low stringency cycles were designed to encourage random priming, and the high stringency cycles were designed to further amplify those randomly produced DNA fragments. A 50 μl DOP-PCR reaction was prepared with 2x GoTaq Green master mix (Promega, Madison, WI) so that resulting fragments had a poly A tail and were easy to clone into an entry vector. Fragments were visualized on a 0.8% agarose gel. All microbial library fragments from the four microbial cultures tested that produced a wide spread band on the gel were combined. Library fragments were generated with the M set primers (M1, M2, M4 and M5) and Rand3 primer as described by Freedman (2014). Pure Culture 1 DOP-PCR reactions using M1, M2, and M3 primers were combined for cloning into the DON sensitive yeast strains. Mixed Culture 1 DOP-PCR reactions using all primers were combined for cloning into the DON sensitive yeast strains. Mixed Culture 2 DOP-PCR reactions using M1 and M2 primers were combined for cloning into the DON sensitive yeast strains. Mixed Culture 3 DOP-PCR reactions using M1, M2, M5, and Rand3 primers were combined for cloning into the DON sensitive yeast strains.

2.2. Yeast strains

Saccharomyces cerevisiae strain R2802 (PDR5 leu2 ura3-52 met5) (Meyers et al., 1992) was used as the parental yeast strain to create a DON sensitive yeast strain as a tool to screen for microbial DNA fragments containing functional DON detoxifying/modifying enzymes or proteins. S. cerevisiae strain R2802 was kindly provided by J. Golin, The Catholic University, Washington, DC. A DON sensitive yeast strain (DSY) was created as described in Abolmaali et al. (2008). Briefly, PDR5 and PDR10 were deleted from wild-type RW2802 (these genes encode for ATP-binding cassette (ABC) multidrug resistance transporter proteins). This strain was then tested for sensitivity to 100 μg/mL DON. DSY5 as well as the parental yeast strain RW2802 were cultured in 2 mL of yeast peptone dextrose (YPD) media for 24 h at 28 °C. 10 μl samples of DSY5 (propanol), to test the hypothesis that 4D is a DON transporter. The specific objectives of this study were to: (1) generate a library of microbial DNA fragments from DON-tolerant microorganisms described in Wilson et al. (2017) using degenerate oligonucleotide primed polymerase chain reaction (DOP-PCR); (2) screen the library of microbial DNA fragments in a DON-sensitive yeast strain for their ability of provide resistance to DON, either by modification or transport from the cell; (3) identify fragments capable of modifying or transporting DON; and (4) conduct experiments to demonstrate the role of a putative transporter (4D) to transport DON out of yeast cells.
and RW2802 were assayed in triplicate in a 96 well plate in 200 μL of YPD media supplemented with 100 μg/mL DON, 100 μg/mL de-epoxy-DON (DED), 50 μg/mL DON and 50 μg/mL DED, and YPD media without any toxins. The yeast was incubated for 24 h at 28 °C with shaking. Then, optical density (OD<sub>600</sub>) measurements were taken with a plate reader to measure growth.

2.3. Cloning into DON sensitive yeast plasmid

Vector pCR8/GW/TOPO TA (Life Technologies, Carlsbad, CA) was combined with 5 μl of the microbial library fragments (described previously) and incubated for 30 min at room temperature before transforming into 50 μl of Clonetech® stellar competent cells (Clonetech Laboratories, Mountain View, CA). Cells were pooled and grown in 2 mL of lysogeny broth (LB) media with 50 μg/L spectinomycin. Plasmid DNA in pCR8 was recombined with pYesDEST-52 by combining 150 ng of plasmid with 50 ng of the pooled pCR8 library DNA using the Invitrogen Gateway LR Clonase II kit (Thermo Fisher Scientific, Waltham, MA) and incubated overnight at 25 °C. Aliquots of 5 μl of the transformation mixture were used to transform E. coli ccdB competent cells. Transformed cells were grown in 2 mL of LB media with ampicillin (100 μg/L). Plasmid DNA was purified using a GeneJet miniprep kit (Thermo Fisher Scientific, Waltham, MA) for transformation into DSYS. We also included a transformation in the DSYS with the empty plasmid pYesDEST-52. This yeast strain served as a control in the screening.

To create the positive control, Pdr5 was amplified from the extracted DNA from the parental yeast strain RW2802 with designed primers Pdr5 full For (5’-TGG CCT GTG TCG AAA GAC TT 3’) and Pdr5 full Rev (5’-ACG CAC CTA TAT GTA GTG ATT ATG T 3’). Hot Start Q5 High Fidelity Master Mix 2X (BioLabs) was used with 2 μl of template (DNA extracted from parental yeast) in a 50 μl PCR reaction. The parameters used in the thermal cycler were: 1 cycle denaturation 98 °C for 30 s, 35 x cycle denaturation 98 °C for 10 s, 35 x cycle annealing 53 °C for 30 s, 35 x cycle extension 72 °C for 2 min 30 s, 1 cycle annealing 54 °C for 45 s, 35 x cycle extension 72 °C for 3 min, 1 x cycle 72 °C for 5 min, hold 4 °C. The amplified Pdr5 gene was then purified with Monarch PCR & DNA Cleanup Kit (BioLabs #T1030G) and treated to have incorporated a poly A tail, in order to be used in TOPO TA cloning (Life Technologies, Carlsbad, CA). The addition of the poly A tail was made by Taq Polymerase 5 U/ul (BioLabs) in a 5 mL reaction of: 1 μl DNA template (purified Pdr5 gene) + 0.2 μl + 0.03 μl dATP + 0.5 μl standard Taq buffer 10x + at 72 °C for 15 min in thermal cycler. TOPO vector (with Pdr5) was recombined with pYesDEST-52 using the Invitrogen Gateway LR Clonase II kit (Thermo Fisher Scientific, Waltham, MA) and incubated at 25 °C. Aliquots of 5 μl transformation mix were used to transform into 50 μl vials of E. coli TOP 10 competent cells and grown in 2 mL of LB media with ampicillin (150 μg/L) selection. Pdr5 gene was identified inside the PyesDEST52 by sequencing the plasmids with universal primer T7 Promotor (5’ TAA TAC GAC TCA CTA TAG GG 3’) and the designed Pyes Primer Reverse 5’ TAG GGA TAG CTC TCT TTG 3’. GoTaqR Green Master Mix (Promega M712) was used with 0.5 μL of plasmid in a 20 μl PCR reaction. The parameters used in the thermal cycler were: 1 × cycle denaturation 95 °C for 2 min, 35 x cycle denaturation 95 °C for 45 s, 35 x cycle annealing 54 °C for 45 s, 35 x cycle extension 72 °C for 3 min, 1 × cycle 72 °C for 5 min, hold 4 °C. The fragment sequences then were queried against the NCBI nr (non-redundant) database to identify similar structures in the database. Accession numbers were obtained from GenBank following submission using the BankIt submission tool at NCBI (ncbi.nlm.nih.gov).

DON concentrations following the screen were measured by GC/MS following standard laboratory protocols (Wilson et al., 2017). Aliquots of 50 μL of each culture were dried down in a glass tube using compressed air in a nitrogen evaporator set at 55 °C. Dried samples were then derivatized at room temperature with a mixture of 99 μL of N-tri-methylsilylimidazole (TMSI) and 1 μL of trimethylchlorosilane (TMCS) for 10 min. Then, 500 μL of isooctane containing 0.5 μg/g of mirex (Sigma-Aldrich, St. Louis, MO, USA) was added to the glass tube, followed by the addition of 500 μL of water to quench the reaction. Samples were vortexed thoroughly. From the top organic layer, 150 μL was transferred to chromatography vials for GC/MS analysis.

An Agilent 6890/5975 system was used for GC/MS analysis operating in selected ion monitoring (SIM) mode. An autosampler in split less mode injected 1 μL of each sample onto an HP-5MS column (0.25 mm inner-diameter, 0.25 μm film thickness, 30 m length) to detect DON. The inlet temperature was set at 280 °C with a column flow rate of 1.2 mL/min using helium. The initial column temperature was held at 150 °C for 1 min, increased to 280 °C at a rate of 30 °C/min, and held constant for 3.5 min. A post-run of 325 °C for 2.5 min was used to clean the column. DON was detected in SIM mode at a mass/charge ratio of 512.3 and had reference ions at 422.4 and 497.3. Mirex (hexachloropentadiene dimer) was used as an internal standard to check the quantitative precision of the instrument and was detected in SIM mode at a mass/charge ratio of 271.8 and had a reference ion of 275.8 (Mirocha et al., 1998). A quadratic regression model was used to quantify DON with standards (Romer Labs, Austria and Sigma-Alrich, St. Louis, MO, USA) at concentrations of 0.05, 0.10, 0.25, 0.5, 1.0, 2.50, 5.0 and 10.0 μg/mL.

2.5. Inhibition experiments

A series of inhibition experiments were conducted to compare OD<sub>600</sub> measurements of the DSYS transformed with the fragments encoding putative transporters, and DSYS transformed with Pdr5 (as a positive control). Propanol (1.5%) was used as an efflux pump inhibitor for DON (Gasková et al., 2013). These experiments were performed on 96 well-plates with the yeast growing on induction media (Khatibi et al., 2013) supplemented with 40 ppm DON. Yeast was harvested in exponential phase in SD-U, to ensure the expression of Pdr5 (Mannun et al., 2004) and other putative transporters. Yeast cultures were then diluted in selective media to give an OD of approximately 0.05, and then 10 μL of this yeast suspension were inoculated in the 96 well-plate to a final volume of 200 μL per well. The inhibition experiments on the plates were incubated for 20 h at 28 °C and 140 rpm.

2.6. Statistical analyses

An analysis of variance (ANOVA) was used to test for significant differences between treatments (selective media, DON, Propanol, DON and propanol) in the same yeast; and between yeast strains
(DSY5_emptyPyesDEST52, DSY5_Pdr5 and DSY5_4D) concerning a single treatment (the growth in DON compared to the growth in media only). The ANOVA test was running as a one-way analysis of variance using the software Minitab 19 (Minitab, Inc.).

3. Results

3.1. Screening of yeast transformants in the presence of 100 ppm DON

Four yeast transformants (4D, 5D, 7D and 12B) were able to grow significantly better than the DON-sensitive parent strain in the presence of 100 ppm DON (Fig. 2) \( (P < 0.05) \). DON concentrations of the yeast transformants were similar to the parent yeast sensitive strain (98.9% of the total DON for DSY, 89% for DSY_4D, 91% for DSY_5D, 90% for DSY_7D, and 86% for DSY_12B), suggesting that the fragments represented potential transporters that were enabling the yeast to survive in the presence of the toxin.

3.2. Sequencing of microbial DNA fragments

Transformants that were able to grow in the presence of DON were plated on a selective medium, and the cloned microbial DNA fragments were sequenced. BLAST queries of one microbial DNA fragment (4D)
showed a high degree of similarity to an ABC transporter. New primers were designed in order to get the full sequence (4D-1-For: 5' TGCTGGGCAATTACCAAGGCG 3'; 4D-2-For: 5' GTGCAAGGCGCCATGAACGC 3'; 4D-1-Rev: 5' ACGCCAGCAGCTTCCAGGAG 3'; 4D-2-Rev: 5' TGGCGTATAGGGGGCGCTGC 3'). The sequence of 4D has been deposited in GenBank, accession #MN536485.4D contains 13 open reading frames (ORFs) and, although the majority of them correspond to hypothetical proteins, one has 84% identity with an ABC transporter from Bordetella bronquiseptica strain FDAARGOS_176 (sequence ID in NCBI: CP014013.2).

3.3. Inhibition experiments

A series of screening and inhibition assays were conducted with a transport inhibitor (propanol), to test the hypothesis that 4D is a mycotoxin transporter. The ability of yeast transformants expressing 4D to transport DON was inhibited by the addition of propanol for two independent experiments (Fig. 3). Moreover, yeast transformants expressing a known efflux pump (PDR5) showed similar trends in propanol transport inhibition compared to 4D for two independent experiments (Fig. 3).

3.4. DON measurements following inhibition experiments

DON concentrations did not change for yeast transformants expressing 4D for two independent experiments. No DON was found in the treatments without DON (media and propanol) (Fig. 4), and DON concentrations did not change significantly in any treatment containing DON (e.g., DON, DON + propanol) for two independent experiments ($P < 0.01$).

4. Discussion

New approaches are needed to detoxify or remove the mycotoxin deoxynivalenol in feed and food. In this study, microbial DNA fragments were generated from a library of DON-tolerant microorganisms described in Wilson et al. (2017) using DOP-PCR. These fragments were screened in DON-sensitive yeast strains for their ability to modify or transport DON. BLAST queries of one microbial DNA fragment (4D) showed a high degree of similarity to an ABC transporter. To our knowledge, this is the first report of a mycotoxin transporter being discovered from a pool of unknown microbial DNA fragments. This work has important implications for the bioprospecting of unique enzymes from unknown pools of microorganisms isolated from the environment.

To test the hypothesis that 4D is a mycotoxin transporter, a series of screening and inhibition assays were conducted with a transport inhibitor (propanol). DON concentrations did not change when transformants expressing 4D were grown in the presence of DON. The ability of yeast transformants expressing 4D to transport DON was inhibited by the addition of propanol. Moreover, yeast transformants expressing a known efflux pump (PDR5) showed similar trends in propanol transport inhibition compared to 4D. ABC transporters, such as PDR5, are ubiquitous in many different groups of microorganisms (Taglicht and Michaelis, 1998), and some act as efflux pumps for a wide spectrum of substances (Taglicht and Michaelis, 1998). PDR5 has been recently found to be actively produced even under conditions of nutrient limitation, because it is important for the survival in the presence of toxic compounds that can be pumped out of cells (Rahman et al., 2018). The relative expression of 4D under nutrient-limiting conditions is unknown, and was beyond the scope of the present study. Further research is warranted to examine the potential for 4D to transport mycotoxins other than deoxynivalenol. We don’t currently know the level of specificity of 4D for DON, and comparisons with the parent DON-sensitive yeast strain in the screening experiments suggest that there may be some cost to expressing the transporter. Our work extends previous studies that have demonstrated the potential of microbial strategies in DON reduction.

The transporter 4D, along with other candidates from our library of microbial DNA fragments, could be used to develop transgenic plants to limit DON accumulations in seeds. For example, resistance of tobacco plants to Fusarium was increased by the insertion of PDR5 (Mitterbauer and Adam, 2002).

Declaration of competing interest

The authors declare no conflict of interest.

CRediT authorship contribution statement

Celia Jimenez-Sanchez: Writing - review & editing. Nina Wilson: Writing - review & editing. Nicole McMaster: Writing - review & editing. Dash Gantulga: Writing - review & editing. Benjamin G. Freedman: Writing - review & editing. Ryan Senger: Writing - review & editing. David G. Schmacle: Writing - review & editing.

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Abbreviations

GC/MS Gas Chromatography Mass Spectrometry
DOP-PCR Oligonucleotide Primed Polymerase Chain Reaction

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