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CLONING AND HETEROLOGOUS EXPRESSION OF CANDIDATE DON-INACTIVATING UDP-GLUCOSYLTRANFERASES FROM RICE AND WHEAT IN YEAST

ABSTRACT

Fusarium graminearum and related species causing *Fusarium* head blight of cereals and ear rot of maize produce the trichothecene toxin and virulence factor deoxynivalenol (DON). Plants can detoxify DON to a variable extent into deoxynivalenol-3-O-glucoside (D3G). We have previously reported the DON inactivating glucosyltransferase (UGT) AtUGT73C5 from *Arabidopsis thaliana* (Poppenberger *et al*, 2003). Our goal was to identify UGT genes from monocotyledonous crop plants with this enzymatic activity. The two selected rice candidate genes with the highest sequence similarity with *AtUGT73C5* were expressed in a toxin sensitive yeast strain but failed to protect against DON. A full length cDNA clone corresponding to a transcript derived fragment (TDF108) from wheat, which was reported to be specifically expressed in wheat genotypes containing the quantitative trait locus Qfhs.ndsu-3BS for *Fusarium* spreading resistance (Steiner *et al*, 2009) was reconstructed. Only cDNAs with a few sequence deviations from TF108 could be cloned. However, toxin sensitive yeast strains expressing this wheat UGT cDNA did not show a resistant phenotype. The main difficulty in generating full length cDNAs for functional validation by heterologous expression in yeast is the enormous number of the UGT superfamily members in plants, with 107 UGT genes plus some pseudogenes in *Arabidopsis thaliana* and about 150 putative UGT genes in grasses. We conclude that neither sequence similarity nor inducibility are good predictors of substrate specificity.

Key words: deoxynivalenol, *Fusarium graminearum*, phase II detoxification, rice, UDP-glucosyltranferase, wheat

INTRODUCTION

DON inhibits eukaryotic protein biosynthesis and is a major virulence factor of *Fusarium graminearum*. The molecular basis of the resistance to DON in plants is the ability of a resistant cultivar to detoxify DON into D3G (Lemmens *et al*, 2005). A gene encoding a DON-detoxifying UGT from *Arabidopsis* was previously identified based on the toxin-resistance phenotype conferred by expression of the cDNA in yeast (Poppenberger *et al*, 2003). In experiments with DON challenged roots and hypocotyls of rice we have obtained evidencethat this detoxification mechanism also exists in the monocot crop plant.

Candidate genes from crop plants were selected previously based on differential expression in cultivars differing in *Fusarium* resistance or due to upregulation during *Fusarium* infection or treatment with DON (e.g. Steiner *et al*, 2009; Desmond *et al*, 2008). Steiner *et al* have investigated the expression profile of wheat cultivars and doubled haploid lines differing in two *Fusarium* resistance QTLs using the cDNA-AFLP method. One differentially expressed transcript derived fragment (TDF108) corresponded to a conserved part of a UGT gene. This UGT is therefore an attractive candidate for a DON detoxification gene.

material and methods

Plant material and DON glycosylation assay

Surface sterilized seeds of the rice cultivar IR24 were grown for two weeks in Magenta boxes in a controlled environment of 16 h/8 h white light/dark cycle at 22 °C. Root and shoot parts were cut from the seedling, sliced into 5 mm pieces and incubated with 3 µl of DON solution (180 mg/L) per mg fresh weight for 16 hours. DON and D3G quantifications were performed using LC-MS/MS as described previously (Berthiller *et al*, 2009).

Isolation and cloning of two rice UGTs from genomic DNA

The genomic sequences of UGTs Os01g0176000 and Os01g0176100 consist of each two exons. We amplified the C-terminal exons with primers annealing about 20 bp up- or downstream of the respective coding regions:

Os01g0176000: CCTAAGAACTTG GTCCAACTATCTCGCAG

and

GGATGAAAATCAGGGTAAGCACAATAGG,
Os01g0176100: TCTTAAGAACTGCCGGTTGGATGTGT

and

AGGTACGATAG TATCCTCCAATTCATTCTTTCA.

The generated fragments were subcloned into pCR4-TOPO, released with EcoRI, requiring a partial digest in case of Os01g0176100 due to an internal restriction site, and ligated into the EcoRI linearized yeast expression vector pADH-FW (P$_{ADH1}$, 2µ, *LEU2*, AmpR, Mitterbauer *et al*, 2002) re-

sulting in pWS1572 (exon 2 of Os01g0176000, 994 bp) and pWS1653 (exon 2 of Os01g0176100, 994 bp). The correct orientation was confirmed by sequencing. We tried without success to capture the exons 1 with different primer combinations. Hence, synthetic fragments comprising the exons 1 (Os01g0176000, 506 bp; Os01g0176100, 512 bp) flanked by overlapping regions of at least 50 bp for homologous recombination in yeast were custom synthesized (Mr. Gene GmbH, Regensburg, Germany) and provided in pMA plasmids (pWS1930: Os01g0175900, 611 bp; pWS1931: Os01g0176000, 617 bp). The recombination sites upstream resemble part of the *ADH1* promotor and the MCS, downstream the overlapping region provides a seamless transition from exon 1 to exon 2. Yeast strain YZGA515 (a derivative of YPH499, Poppenberger *et al*, 2003) was cotransformed with the released exon 1 constructs (pWS1930: AscI/PacI; pWS1931: HindIII/KpnI) and the respective exon 2 constructs in pADH-FW linearized with BamHI behind the promoter sequence. Transformants were selected in SC-LEU medium. Plasmid DNA with the full-length ORFs (pWS1957: Os01g0176000 and pWS1959: Os01g0176100) was recovered and transformed into *E. coli* for sequencing.

Reconstruction of a candidate wheat UGT from a cDNA-AFLP fragment

Dissected spikelets from the resistant cultivar CM82036 after *Fusarium* infection were used for the generation of cDNA (Steiner *et al*, 2009). The expression was tested by performing real-time RT-PCR using the realplex2 S cycler (Eppendorf, Hamburg, Germany) and primers:

      TDF108_F (TGGCGGCATGAGACTTTG)

and

      TDF108_R (GCTTACGGAGCGAGGATTAC).

For cloning of the full-length cDNA corresponding to TDF108 the 'Smart RACE' cDNA kit (Clontech, Mountain View, CA) was used according to manufactures instructions. Primers:

      (GTGAGCAATGCCCGGGCCACATCGTC

and

      TGGGTGGAACTCCGTCCTAGAGGCAGTGGC)

were designed to discriminate all but the closest matching ESTs. The generated 5' and 3' extended PCR products were subcloned into pCR4-TOPO vectors (5' pWS1460, 3' pWS1478), sequenced and assembled into a full-length clone (pWS1540) by overlap extension PCR using external primers providing a 5' HindIII (AT<u>AAGCTT</u>GCCATCATGGCTTCTTCTATCACTAGCAG) and a 3' NotI (GG<u>GCGGCCGC</u>AAGCTAGCTAGTTCC) site for ligation into yeast expression vector pYAK7 (P$_{ADH1}$-c-myc, 2μ, *LEU2*, AmpR), yielding pWS1840. The resulting cDNA was sequenced and compared to the EST database.

Expression of candidate genes in yeast and phenotypic testing

The UGT constructs were used to transform yeast strains YZGA515 (*pdr5 pdr10 pdr15 ayt1*) or YZDL112 (isogenic to YZGA515 but *trp1*) in case of the rice UGTs. Exponentially growing cultures were diluted to an OD_{600} of 0.05 and 3 µl of the suspensions were spotted on YPD plates containing increasing concentrations of DON and other trichothecenes. Extracts were prepared from 5 OD600*ml for immunodetection using a mouse anti-c-Myc antibody (1:5000, clone 9E10), which binds to the respective N-terminal epitope tag of the UGTs cloned into the pYAK7 vector (Poppenberger *et al*, 2003).

RESULTS

Identification of putative orthologs to AtUGT73C5 and TaUGT3 in O. sativa

We found that the rice cultivar IR24 had very high ability to convert DON into D3G (data not shown). Consequently the rice genome has to contain one or several genes encoding DON-inactivating UGTs. We first searched the sequenced genome for UGTs with the highest sequence similarity to the DON inactivating *AtUGT73C5* from *Arabidopsis* (Poppenberger *et al*, 2003). We identified a cluster of four UGT genes, Os01g0175900 (1506 bp), Os01g0176000 (1500 bp), Os01g0176100 (1506 bp) and Os01g0176200 (1569 bp), on chromosome 1. Another indication for a possible role of this cluster in DON or *Fusarium*-resistance was provided by Boddu *et al.* (2007). They identified several UGTs in barley responding to *Fusarium* infection based on expression analysis with the Barley1 Affymetrix GeneChip. Among these, *HvUGT5876* shows the highest similarity to *AtUGT73C5* and the rice genes in the cluster. The same rice genes were also identified when the sequence of *TaUGT3*, which was found upregulated by DON in a wheat microarray experiment (Lulin *et al*, 2009), was used as a query sequence. The predicted proteins of the cluster members show high amino-acid sequence identity between each other (up to 87.6 %) and with *HvUGT5876* (73 %) and *TaUGT3* (73 %).

Phenotypic testing of Os01g0176000 and Os01g0176100 in yeast

The second exons of Os01g0176000 and Os01g0176100 were amplified from rice genomic DNA and cloned behind the constitutive *ADH1* promoter in yeast expression vector pADH-FW. We acquired the respective N-terminal exons as custom synthesized DNA fragments, flanked by about 50 bp of overlapping sequence identical to the pro-

moter region of ADH1 and the respective UGT exons 2 sequences. Complete cDNAs were assembled by homologous recombination in yeast. The recovered plasmids were sequenced and introduced into strain YZDL112 for phenotypic testing. Suspensions of log phase transformants spotted onto plates containing increasing amounts of DON showed no increased DON resistance compared to strains transformed with the empty vector (except for the positive control expressing *AtUGT73C5*, data not shown).

Construction and functional testing of full-length cDNAs related to TFD108

Steiner *et al.* (2009) have published the results of a cDNA-AFLP study comparing *Fusarium* inoculated resistant (CM82036) and susceptible (Remus) wheat. A transcript derived fragment unique for the resistant cultivar challenged with *Fusarium* was identified as part of a UGT gene (TDF108, EX982036, 352 bp). It corresponds to the highly conserved C-terminal signature motif, which is responsible for binding the UDP-sugar co-substrate. Real-time RT-PCR was performed to independently test the very strict expression observed in the cDNA-AFLP assay, showed that the inducibility of the investigated gene was 133-fold in the resistant cultivar CM82036 and 73-fold in the sensitive cultivar Remus. In two double haploid lines from the cross CM82036/Remus mapping population, which are about 70 % identical in the rest of the genome, but differ in the resistance loci Qfhs.ndsu-3BS and Qfhs.ifa-5A, the induction was 53 and 35-fold, respectively.

Prior to cloning the full length cDNA we searched for and identified 50 wheat ESTs with high similarity to TDF108. However, none was completely identical to TDF108. Despite numerous attempts we were unable to generate the exact TDF108 sequence from CM82036 cDNA but obtained two sequences (pWS1380 and pWS1387) that differed by a few nucleotide changes from the original TDF108, (see alignment in Fig. 1a). We assembled the RACE-PCR products resembling the N- and C-terminal ends of pWS1380 by overlap extension PCR, backed by a model ORF, that was generated from ESTs (CN013007, BE585608, CN011404 and CK206211; Fig. 1b), which cover almost the entire ORF. Reconstruction of the second UGT failed as the PCR products contained a frameshift (Fig. 1a) and EST data was inconclusive. The full-length cDNA was cloned behind the c-Myc epitope tag in the expression vector pYAK7, and strain YZGA515 was transformed with the resulting plasmid (pWS1840). Again no increased DON resistance of transformants was observed (Fig. 1c), also not to 15-acetyl-DON, nivalenol or T2 toxin, although immunoblotting confirmed the high expression of the gene (Fig. 1d).
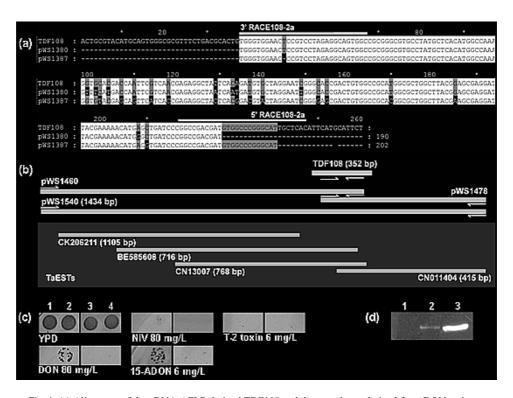
Fig. 1. (a) Alignment of the cDNA-AFLP derived TDF108 and the two clones derived from DON resistant cultivar CM82036 cDNA. (b) Generation of the complete UGT gene based on the sequence of pWS1380 and comparison to the EST database. (c) Suspensions of toxin sensitive yeast strains containing the empty vector (1), expressing the DON inactivating *AtUGT73C5* (2) or individual transformants with the candidate UGT corresponding to TDF108 from wheat (3, 4) spotted on plates containing DON, nivalenol (NIV), 15-acetyl-deoxynivalenol (15ADON) or T-2 toxin. (d) Expression was confirmed by immunoblotting (same numbering)

DISCUSSION

We have cloned and tested candidate UGT genes from rice and wheat for the ability to detoxify DON into D3G and found that neither sequence similarity nor inducibility by Fusarium were useful indicators for UGT gene function. Both candidate UGT genes from rice showed to be inactive against DON, albeit their high similarity to the DON inactivating *UGT73C5* from *Arabidopsis*. We also expressed the putative orthologs *HvUGT5876* and *TaUGT3* in yeast (Schweiger *et al*, 2010) and also these genes did not confer toxin resistance. The assembly of full length cDNA sequences from highly conserved C-terminal parts of the UGTs from crop plants lacking whole genome sequences showed to be extremely difficult. The enormous size of the UGT superfamily, and especially the presence of clusters of highly similar genes and of paralogous loci

from genome duplications or fusion of ancestral genomes, such as in hexaploid wheat, complicate primer design for cloning and RT-PCR. Since we did not obtain the identical UGT corresponding to TDF108, but only a very similar one, we can not formally exclude the possibility that the authentic gene product would be active against DON, but it seems rather unlikely. The recent identification of a barley DON-glucosyltransferase (Schweiger *et al*, 2010) which is located in a completely different branch of the superfamily, should facilitate the cloning and functional testing of candidates from crop plants such as wheat or maize.

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