Detoxification of the Fusarium Mycotoxin Deoxynivalenol by a UDP-glucosyltransferase from Arabidopsis thaliana*

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Plant pathogenic fungi of the genus Fusarium cause agriculturally important diseases of small grain cereals and maize. Trichothecces are a class of mycotoxins produced by different Fusarium species that inhibit eukaryotic protein biosynthesis and presumably interfere with the expression of genes induced during the defense response of the plants. One of its members, deoxynivalenol, most likely acts as a virulence factor during fungal pathogenesis and frequently accumulates in grain to levels posing a threat to human and animal health. We report the isolation and characterization of a gene from Arabidopsis thaliana encoding a UDP-glycosyltransferase that is able to detoxify deoxynivalenol. The enzyme, previously assigned the identifier UGT73C5, catalyzes the transfer of glucose from UDP-glucose to the hydroxyl group at carbon 3 of deoxynivalenol. Using a wheat germ extract-coupled transcription/translation system we have shown that this enzymatic reaction inactivates the mycotoxin. This deoxynivalenol-glucosyltransferase (DOGT1) was also found to detoxify the acetylated derivative 15-acetyl-deoxynivalenol, whereas no protective activity was observed against the structurally similar nivalenol. Expression of the glucosyltransferase is developmentally regulated and induced by deoxynivalenol as well as salicylic acid, ethylene, and jasmonic acid. Constitutive overexpression in Arabidopsis leads to enhanced tolerance against deoxynivalenol.

A complex of closely related species of the genus Fusarium is responsible for destructive and economically very important diseases of cereal crops (Fusarium head blight of wheat and barley) and maize (Fusarium ear rot). In years with climatic conditions that favor the development of the fungi, these infections can reach epidemic proportions (1). Diseases caused by Fusarium do not only severely reduce yield but also result in contamination of grain with unacceptably high amounts of mycotoxins, a problem of world-wide significance. A toxin class of particular concern to human and animal health is the trichothecces, sesquiterpenoid epoxides, which are potent inhibitors of eukaryotic protein synthesis. More than 180 compounds of this class have been isolated from natural sources, predominantly from Fusarium species (2). Depending on the concentration and the substitution pattern of the trichotheccene, either translational initiation, elongation, or termination are preferentially inhibited (3).

Fusarium graminearum and Fusarium culmorum are the two most common causative agents of Fusarium head blight of cereals. F. graminearum lineages present in Europe and North America predominantly produce deoxynivalenol (DON) as well as the acetylated derivatives 3-acetyl-deoxynivalenol (3-ADON) and 15-acetyl-deoxynivalenol (15-ADON), whereas producers of nivalenol (NIV), which contains one additional hydroxyl group (Fig. IA), predominate in Asia (4). While the toxicity of these trichotheccenes is well studied in animal systems (5), fairly little is known about differences in phytotoxicity. In contrast to animal cells, NIV and T-2 toxin are less toxic than DON to wheat (6), which may indicate significant differences in uptake or metabolism of particular trichotheccenes. Animal exposure to DON (also known as vomitoxin) has numerous adverse health effects with neural and immune system being the most sensitive targets (7). In contrast to high doses of DON, which inhibit antibody production, low doses of DON act synergistically with bacterial lipopolysaccharide to stimulate proinflammatory processes (7). To protect consumers, the United States Food and Drug Administration has established advisory levels for food. The European Community has also recently recommended action levels for DON (8).2

The role of DON in plant disease is still a matter of discussion (9), but most of the evidence supports the hypothesis that it functions as a virulence factor. Fusarium mutants with a disrupted trichodiene synthase (Tri5) gene, involved in trichotheccene biosynthesis, are still pathogenic. However, they exhibit reduced virulence on wheat (10); they are unable to spread from the infection site (11). DON can move ahead of the fungus in infested plants, suggesting a role in conditioning host plant defense (11).

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1 The abbreviations used are: DON, deoxynivalenol; 3-ADON, 3-acetyl-deoxynivalenol; 15-ADON, 15-acetyl-deoxynivalenol; NIV, nivalenol; UGT, UDP-glucosyltransferase; DOGT1, DON-glucosyltransferase 1; GUS, β-glucuronidase; SA, salicylic acid; JA, jasmonic acid; ACC, 1-aminoacyclopropylcarboxylic acid; fw, forward; rv, reverse; Col-0, Columbia-0; HPLC, high pressure liquid chromatography; LC, liquid chromatography; MS, mass spectrometry; GST, glutathione S-transferase.
2 www.mykotoxin.de/Dokumente/Codex%20DON%202012.02.pdf.
concentration of DON, which occurred in *Fusarium*-infected wheat in the field (15), suggests that the toxin may be metabolized. Two wheat cultivars differing in *Fusarium* resistance also differ in their ability to form a DON metabolite, suspected to be a glycocide (16). Sewald et al. (17) used radish-leaded DON to show that it was primarily conjugated to 3-β-glucopyranosyl-4-deoxynivalenol in a maize suspension culture. No plant enzymes capable of modifying the trichothecene have been described so far.

Genome sequencing projects have revealed the existence of a vast number of genes in plants that code for putative UDP-glycosyltransferases (UGTs), predicted to conjugate small molecules. For instance, *Arabidopsis thaliana* has been shown to harbor more than 100 members of this multigene family (18) whose functions are largely unknown. Here we report the cloning of an *Arabidopsis* UGT by functional expression in yeast that is able to inactivate the *Fusarium* mycotoxin DON.

**EXPERIMENTAL PROCEDURES**

**YEAST STRAINS**—The yeast strains used in this work are derived from YPH499 (Mat a, ade2-101oc, his3-200, leu2-1, lys2-801a, trpl-1Δ, ura3-52) (19). Standard techniques (20, 21) were used to inactivate genes encoding ATP-binding cassette transporters.4 The relevant genotype of strain YZGA52 is pdr5Δ:TRP1, pdr10Δ:hisG snq2::hisG, yor1Δ:hisG. YZGA515 (pdr5Δ:TRP1, pdr10Δ:hisG, pdr15Δ:loxP::KanMX-losP, ayt1Δ::URA3) was constructed by disruption of the acetyltransferase *AYTI* in strain YHW1615K.5 In the ayt1Δ::URA3 construct nucleotides 343-1022 of the *AYTI* reading frame are replaced by a 1.1-kb HindIII fragment.

**PLANT MATERIAL AND GROWTH CONDITIONS**—*A. thaliana* experiments were conducted with the wild-type ecotype Columbia-0 (Col-0). For propagation, seeds were sterilized, plated on standard Murashige and Skoog growth medium (22) supplemented with 1.0% sucrose and 1.0% phytagar (Invitrogen), and subjected to a 2-day dark treatment at 4 °C to synchronize germination. The seedlings were grown for 2 weeks in a controlled environment of 16 h/8 h light-dark cycle (140 μmol m−2 s−1 white light) at 22 °C before they were transferred to soil and grown at 20 °C and 55% humidity under continuous white light.

*A. thaliana* cDNA Library Screen in Yeast—The ATP-binding cassette transporter-deficient *Saccharomyces cerevisiae* strain YZGA462, which is hypersensitive to DON, was transformed with an *A. thaliana* cDNA library constitutively expressed under the control of the phosphoglycerate kinase (PGK1) promoter (23). A total of 109 transformants were selected on minimal medium lacking uracil and transferred to medium containing 180 ppm DON (kindly provided by Marc Lemmens), a dose sufficient to completely inhibit growth of yeast transformed with the empty library plasmid. Colonies that showed resistance were isolated, and the plasmid dependence of the phenotype was tested by plasmid DNA preparation and retransformation of YZGA452. The NotI fragment containing the cDNA insert of the candidate was subcloned into pBlueScript SKII+ (Stratagene) and sequenced.

**CONSTITUTIVE EXPRESSION AND IMMUNODETECTION OF THE DON-GLYCOSYLTRANSFERASE 1 (DOGT1) IN YEAST**—The intronless open reading frame of *DOGT1* (GenBank accession AF006034) was used as a jumping-off point to isolate the corresponding cDNA clone from a *A. thaliana* cDNA library.5 PCR products were cloned into the HindIII fragment containing the cDNA insert of the candidate was subcloned into pBluescript SKII+ (Stratagene) and sequenced.

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YPD (1% yeast extract, 2% peptone, 2% dextrose) medium containing increasing concentrations of different trichothecenes. The toxins used were: DON, 3-ADON, 15-ADON, NIV, trichothecin, T-2 toxin, HT-2 toxin, diacetoxyscirpenol, and verrucarin A. With the exception of DON, 3-ADON, and NIV, which were obtained from Biopure Referenzsubstanzen GmbH (Tülln, Austria), mycotoxins were purchased from Sigma and stored at -20 °C dissolved in 70% ethanol.

For immunodetection, the extraction of proteins from yeast cells was performed as described by Egner et al. (25). Western blot analysis was conducted with a primary mouse anti-c-Myc antibody (1:5000, clone 9E10, Invitrogen).

**Synthesis of 3-β-D-Glucopyranosyl-4-deoxynivalenol and 15-β-D-Glucopyranosyl-4-deoxynivalenol**—To obtain reference material for HPLC and TLC, DON-3-glucoside and DON-15-glucoside were synthesized in two-step reactions. In the first step, 15-acytetyl-DON or 3-acytetyl-DON were modified with 1-bromo-1-deoxy-2,3,4,6-tetra-O-acetyl-β-D-glucopyranose (acetobromoglucose) in toluene with CdCO₃ as catalyst to yield the DON-glucoside-acetates (26). Gentle hydrolysis of the acetates to the glucosides was performed in the second step using a strong basic anion exchanger (Dowex 1 × 2–4, 400 Aldrich). After checking the progress of the reaction with TLC (mobile phase: toluene/ethyl acetate, 1:1, v/v), the DON-glucosides were cleaned up using flash chromatography over silica gel with 1-butanol/1-propanol/ethanol/water (2:3:3:1, v/v/v/v). Further purification of the substances was performed with HPLC by means of an RP-18 Aqaulis column (Keystone, Bellefonte, PA) using acetone/methanol/water (10:90, v/v) at 32 °C. The synthesized DON derivatives were characterized in the negative electrospray interface mode. LC-MS/MS analysis was performed on a QTrap LC-MS/MS system (Applied Biosystems, Foster City, CA) equipped with electrospray interface and a 1100 Series HPLC system (Agilent, Waldbronn, Germany). Chromatographic separation was achieved with a 10-cm × 4.6-mm inner diameter, 3-μm Aquarl RP-18 column (Keystone) at 22 °C using methanol/water (28:72, v/v). The flow rate was set to 0.3 ml/min. The electrospray interface was used in the negative ion mode at 400 °C with the following settings: curtain gas (CUR), 20 psi.; nebulizer gas (GS1), 30 psi.; auxiliary gas (GS2), 75 psi.; ion spray voltage (IS), -4200 V; declustering potential (DP), -46 V; entrance potential, -9 V; collision energy (CE), -30 eV; collision-activated dissociation gas (CAD), high; linear ion trap fill time (LIT), 50 ms; quadrupole 3 entrance barrier, 8 V.

**Isolation and Analysis of DON Metabolites in Vivo**—To elucidate the chemical structure of DON metabolites resulting from enzymatic transformation of the mycotoxin by DOGT1, a highly tolerant strain was constructed, and the DON metabolite was extracted from toxin-treated yeast. Transformants were selected on synthetic complete medium (4:1), and sonicated. After centrifugation the supernatant was filtered three times with ice-cold water, extracted in 2.5 ml of methanol/water (28:72, v/v). The flow rate was set to 0.3 ml/min. The electrospray interface was used in the negative ion mode at 400 °C with the following settings: curtain gas (CUR), 20 psi.; nebulizer gas (GS1), 30 psi.; auxiliary gas (GS2), 75 psi.; ion spray voltage (IS), -4200 V; declustering potential (DP), -46 V; entrance potential, -9 V; collision energy (CE), -30 eV; collision-activated dissociation gas (CAD), high; linear ion trap fill time (LIT), 50 ms; quadrupole 3 entrance barrier, 8 V.

**Enzyme Assays**—The glucosyltransferase activity assay mixture contained 1 μg of recombinant GST fusion protein, 10 mM 2-mercaptoethanol, 50 mM Tris-HCl, pH 7.0, 0.5 mM radioactive labeled UDP-[14C]glucose (4.4 × 10⁶ cpm, PerkinElmer Life Sciences), 0.01% bovine serum albumin, and a 1 mM concentration of acceptor substrate (dissolved in Me2SO in 20 mM stock solutions). The reactions were carried out in 20 μl volumes at 30 °C for 2 h, stopped by adding 1 ml of trichloroacetic acid (240 mg/ml), frozen, and then stored at -20 °C.

Analysis of reaction products was performed by TLC. An aliquot of each sample was spotted on a silica gel plate (Kieselgel 60, Merck) and developed with a mixture of 1-butanol/1-propanol/ethanol/water (2:3:3:1, v/v/v/v). The intensity of each radioactive spot was determined using a PhosphorImager (STORM 860 system, Amersham Biosciences). The plates were additionally stained with p-anisalddehyde (0.5% in methanol/HSO₄/acetic acid, 85:5:10, v/v/v).

**Inhibition of Wheat Ribosomes in Vitro**—To analyze whether the 3-β-D-glucopyranosyl-4-deoxynivalenol is less phytotoxic than its aglycone, we used a wheat germ extract-coupled in vitro transcription/translation system (TNT coupled wheat germ extract, T3, Promega). A forming transcription reaction was carried out according to the manufacturer’s instructions (in the presence of either DON, purified DON-glucoside (1, 2, 5, 10, and 20 μM), or water as a control), the activity of the firefly luciferase reporter was determined (luciferase assay system, Promega) using a luminometer (Victor 2, Wallace).

**Plant Treatment with Different Stress Response-related Compounds for Expression Analysis**—For reverse transcription-PCR analysis of mRNA expression of DOGT1 following treatments with DON, salicylic acid (SA), jasmonic acid (JA), and 1-aminocyclopropylcarboxylic acid (ACC) seedlings were grown for 2 weeks on vertical Murashige and Skoog plates (0.8% phytagar) before they were transferred to liquid Murashige and Skoog medium. The plants were incubated for 48 h on an orbital shaker (50 rpm) before adding 5 ppm DON, 200 μM SA, 2 μM ACC, or 50 μM JA. The compounds were kept as stock solutions dissolved either in 70% ethanol or in Me2SO. Ethanol and Me2SO treatments were performed as controls. Plants were harvested at different time points, ground in liquid nitrogen, and stored at -70 °C until RNA extraction was performed.

**Analysis of mRNA Expression of DOGT1 by Reverse Transcriptase PCR**—Total RNA was isolated from plant tissue ground in liquid nitrogen with TriZol reagent as recommended by the manufacturer (Invitrogen). RNA was quantified photometrically and visually on a denaturing RNA gel analyzing 5 μg of total RNA. cDNA was synthesized from 1 μg of total RNA (digested with DNase I) using 500 ng of an 18-mer oligo(dT) and SuperScript reverse transcriptase (Invitrogen). PCR was performed with -2 μl of the 1:20 diluted cDNA using primers that amplify C-terminal fragments of DOGT1 and the UBQ5 control gene: DOGRT-fw, 5'-ATCCGCGGTT-GAAGAGGCT-3'; DOGRT-rv, 5'-TCAATTTGTTGTCTGC-3'; UBQ5-f, 5'-GTCTCTTCTTTGGAAGGT-3'; UBQ5-r, 5'-AACTCCATTGTTAATGCT-3'. To compare relative amounts of transcripts, the samples, DNA fragments of the UBQ5 was first amplified, and normalized sample volumes, based on the amount of products corresponding to the UBQ5 transcripts, were used for PCR.

**Cloning of Plant Overexpression and GUS Fusion Constructs**—For constitutive overexpression of c-Myc-tagged DOGT1 protein in Arabidopsis, the vector pB1P319 was constructed. It is derived from a modified version of the plant expression vector pZZFP22 (27). The promoter Klenow fragment of the constitutive UBQ5 was introduced via the adenylation signal of cauliflower mosaic virus strain CabB D-, came from the vector p2RT, a modified version of pRT100 (28).

The c-Myc-tagged DOGT1 fragment was isolated from Smal + NotI digestion (Klenow-filled) from the yeast expression vector and cloned into Clal + SacI-digested pBlueScript SKI +, which was treated with Klenow enzyme. In the next step, the gene was excised from the resulting plasmid as a Sall + BamHI fragment and inserted in the Xhol + BamHI sites of p2RT. The obtained 2x35S c-Myc-DOGT1 cassette was isolated by PsiI digestion and cloned into the unique PsiI site of pPZFP22 after the multiple cloning site in that vector had been destroyed by digesting the plasmid with EcoRI + Sall, filling the sites
with Klenow, and religating it (p235a). In the resulting vector pBP1319, the 2x35S c-Myc-DOGT1 cassette is oriented in the opposite site direction than the 2x35S gentamycin resistance marker.

For construction of a transcriptional DOGT1-GUS fusion, the GUS vector pZP-GUS.1, which originates from pZP200 and contains the GUS gene from pB1101.1 (inserted by HindIII + EcoRI digestion into the MCS), was used (29). The DOGT1 promoter region was PCR-amplified from genomic DNA using a DNA polymerase with proof reading activity (Fru polymerase, MB1) and specific primers (DOGP-GUS-fw, 5'-GTTAAAACTTACATGCGATTACGGTCTGTTGAAA-TA; DOGP-GUS-rv, 5'-TTCCGATCCTAGGTATTTATCAACCTTAGTGAA-AACTCTC). The resulting product was cloned in-frame with the GUS gene by HindIII + BamHI digestion into the pZP-GUS.1 vector. Constructs were confirmed by DNA sequencing.

Generation and Analysis of Transgenic A. thaliana—For all plant transformations, the recA-deficient Agrobacterium tumefaciens strain UA143 (30), which harbors the helper plasmid pMP90 (31), was used. A. thaliana was transformed applying the floral dip technique (32). The progeny of 15 independent transformants were selected through three generations to obtain homozygous lines.

For immunodetection of c-Myc-tagged DOGT1, about 200–500 mg of plant material were homogenized in liquid nitrogen. 300 μl of extraction buffer (200 mM Tris-HCl, pH 8.9, 200 mM KCl, 35 mM MgCl₂, 12.5 mM EGTA, 15 mM dithiothreitol, 0.6 mM sorbitol) and 15 μl of protease inhibitor mixture (Sigma catalog no. 9599) were added to the still frozen samples, and the mixture was incubated with vigorous shaking for 15 min at 4 °C. After centrifugation (14,000 rpm for 15 min at 4 °C), 200 μl aliquots of the supernatants were transferred into fresh tubes and stored at −20 °C. Equivalent amounts of protein (50 μg) were used for Western blot analysis, which was carried out using primary anti-c-Myc antibody purified from hybridoma supernatant (clone 9E10).

For analysis of DON resistance, seeds of homozygous lines exhibiting high DOGT1 expression and Col-0 as control were germinated on Murashige and Skoog media containing different concentrations of DON (5–30 ppm). Seedlings were grown for 5 weeks before the phenotype was documented. GUS activity was analyzed by staining seedlings or organs of adult plants in 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc) solution for 2–4 h at 37 °C (33).

RESULTS

Isolation of the DOGT1 by Heterologous Expression in Yeast—To identify plant genes that contribute to mycotoxin resistance, an unbiased functional screen based on heterologous expression of cDNAs in yeast was set up. Wild-type S. cerevisiae is highly resistant to DON. To reduce the amount of toxin necessary for the screen, we generated a strain deficient in four ATP-binding cassette transporters, which are to a large extent responsible for pleiotropic drug resistance in yeast (34). Strain YZGA452 (snq1Δ::hisG pdr5Δ::TRP1 pdr10A::HisG yor1Δ::HisG) is hypersensitive to a wide range of different xenobiotic substances and natural products, including DON (data not shown). YZGA452 was transformed with a cDNA expression library of A. thaliana (23) in which cDNAs are constitutively expressed under the control of the yeast phosphoglycerate kinase promoter. Ten million transformants were generated, and diluted pools of transformants were plated on DON-containing medium. After selection of DON-resistant yeast colonies and confirmation of the plasmid dependence of the phenotype, the insert was subcloned and sequenced.

DOG1 is a Member of the UDP-glycosyltransferase Family of Arabidopsis and Exhibits High Similarity to Salicylic Acid- and Wound-inducible UGTs of Other Species—The cDNA insert conferring resistance was 1.75 kb in length and contained an open reading frame of 1488 bp encoding a putative UDP-glycosyltransferase. The identified DOGT1 corresponds to gene UGT73C5 ( locus At2g36800) and belongs to subfamily 73C in group D of A. thaliana UGTs (35). Arabidopsis UGTs constitute a very large gene family that has been divided into 14 distinct groups believed to have originated from common ancestors (18). DOGT1 is located on chromosome II in a cluster with five other members of the subfamily 73C (bacterial artificial chromosome clone F13K3, European Molecular Biology Laboratory (EMBL) accession number AC006282). All six tandemly repeated genes contain no introns and are highly similar to each other (77–89% identity at the amino acid level). The similarity is also very high in the intergenic promoter regions.

A data base search using the deduced DOGT1 amino acid sequence revealed high similarity to glucosyltransferases from tobacco (TOGT1, Ref. 36; I5a and I5b, Ref. 37) and tomato (Tw1, Ref. 38) and to the betanidin 5-O-glucosyltransferase of Dorotheanthes baldiliiformis (40). Expression of the glucosyltransferases from tobacco and tomato has been shown to be elevated following treatment with SA, fungal elicitors, or wounding (36, 38, 39). Two putative, uncharacterized glucosyltransferases from Vigna angularis (ADGT-9) and Oryza sativa, which have similarity to DOGT1, are also included in the amino acid alignment shown in Fig. 2. Regions of high similarity were observed in both N- and C-terminal domains of the sequences. Indicated in Fig. 2 are the hypothetical acceptor substrate binding region (40) and the UGT consensus sequence (35).

The Expression of DOGT1 Is Developmentally Regulated and Induced by DON and Other Stress Response-related Compounds—To investigate whether DOGT1 expression is regulated similarly to that described for the related genes of other plant species, we constructed a reporter by placing the open reading frame of the β-glucuronidase reporter gene behind the DOGT1 promoter (PDGT1-GUS). The tissue-specific expression of the transcriptional GUS fusion was examined histochemically in transgenic Arabidopsis homozygous for the fusion gene. The results shown in Fig. 3 demonstrate that DOGT1 expression is regulated developmentally and is overall rather low. In seedlings, GUS activity was observed to be root-and hypocotyl-specific with the strongest expression in the vascular system, in meristematic tissue of the root tips (in the primary root as well as in lateral roots), and in the vasculature of the hypocotyl right after germination. Staining in the vasculature decreased significantly later in development, and a patchy staining pattern appeared in epidermal root cells. In adult plants GUS activity was detected in late stages of flower development in petals and in abscission zones (Fig. 3A).

Expression of seedlings to either DON (5 ppm or 16.9 μM for 4 h, Fig. 3B) or the ethylene precursor ACC (2 μM for 1 h, not shown) was found to induce PDGT1-GUS expression. No induction of expression of the reporter was detected upon SA treatment (200 μM for 12 h) or treatment with JA (50 μM for 1 h). Semiquantitative reverse transcriptase PCR was used to validate the results obtained from GUS reporter analyses by detecting changes in mRNA levels of DOGT1 after treatment with the same concentrations of DON, SA, ACC, and JA.

As shown in Fig. 3C the results of the reverse transcriptase PCR confirmed that DOGT1 expression was induced by DON as observed previously with the reporter construct. An increase in the amounts of transcript was apparent after only 1 h of incubation with the toxin, reaching a peak after 4 h before declining between 6 and 12 h. After SA treatment, DOGT1 expression was evident at low levels at 4 h and increased slightly by 12 h. It must be noted that the applied 200 μM SA induced expression rather weakly. Jasmonic acid as well as treatment with ACC also led to weak induction of expression of DOGT1 after 1 h of treatment but rapidly declined with no transcript accumulation detectable after 2 h of exposure to the compounds (Fig. 3C).

Phenotypic Determination of the Trichothecene Resistance Spectrum in Yeast—Trichothecene toxicity in animals depends on the hydroxylation pattern as well as on the position, num-

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*N. Malenica, unpublished.*
ber, and complexity of esterifications (5). The basic trichothecene structure and the numbering of its carbon atoms is shown in Fig. 1A. Members of subclass B (e.g. DON and NIV) contain a keto group at carbon 8, while type A trichothecenes (e.g. the highly toxic T-2 toxin produced by Fusarium sporotrichoides) do not. Extremely toxic also are the macrocyclic trichothecenes, like verrucarin A, which contain a macrocyclic ring with ester bonds bridging carbon 4 and carbon 15. Yeast pdr5 mutants are hypersensitive to all the trichothecenes tested so far, allowing us to investigate the ability of DOGT1 and other genes in the cluster to confer resistance to various members of the trichothecenes.

The DOGT1 gene was expressed in yeast strain YZGA515 as a fusion protein tagged with an N-terminal c-Myc epitope (see “Experimental Procedures”). Several independent yeast transformants were spotted on media containing increasing concentrations of various trichothecenes; transformants containing the empty expression vector were used as controls.

As shown in Fig. 1B, DOGT1 had the ability to protect against DON and 15-ADON but did not protect against other tested trichothecenes (data not shown). The following minimal inhibitory concentrations for the strain containing the empty vector were estimated from the results of the plate assays: DON, 30 ppm or 100 μM; 3-ADON, 70 ppm or 207 μM; 15-ADON, 5 ppm or 15 μM; NIV, 30 ppm or 96 μM; trichothecin, 0.01 ppm or 0.03 μM; HT-2 toxin, 20 ppm or 47 μM; T-2 toxin, 2 ppm or 4 μM; diacetoxyscirpenol, 4 ppm or 11 μM; and verrucarin A, 0.05 ppm or 0.1 μM. As expected, we observed no increased resistance to trichothecin, which has no free hydroxyl group. Trichothecin was therefore used as a negative control. It should be emphasized that DOGT1 did not protect against 3-ADON, which differs from DON only in that the C-3 hydroxyl
group is already blocked by an acetyl group. In contrast, the acetyl group at the C-15 hydroxyl group did not interfere with DOGT1 protection against 15-ADON. DOGT1 expression did not protect against NIV, which differs from DON only in a single additional hydroxyl group (see Fig. 1A).

In Vivo and in Vitro Analysis Prove That DOGT1 Catalyzes the Transfer of Glucose from UDP-glucose Specifically to the 3-OH Position of DON—The protection against 15-ADON and the inability to confer resistance against 3-ADON suggest that DOGT1 may catalyze the formation of a DON-3-O-glucoside. To test this hypothesis, we first chemically synthesized DON derivatives with the glucose moiety attached either to the C-3 or C-15 hydroxyl group. The two products were characterized with LC-MS/MS. The DON-3-O-glucoside and DON-15-O-glucoside eluted at 12.43 and 12.68 min, respectively, and the mass spectra of the glucosides showed characteristic differences in their fragmentation pattern (Fig. 4, A and B). While the DON-3-O-glucoside fragmented to an ion of 427.2 m/z under the given conditions, the same ion was not detected with DON-15-O-glucoside. The loss of 30 atomic mass units can be explained by the cleavage of the -CH$_2$OH group at C-6, which is prevented when the hydroxyl group is conjugated with glucose as in DON-15-O-glucoside. Further breakdown (MS/MS/MS) of the DON-glucosides in the linear ion trap showed an almost identical fragmentation pattern to that of the [DON - H]$^+$ ion (295.3 m/z, not fragmented in quadrupole 2), confirming the presence of DON entities in the reaction products.

With these tools at hand we were able to directly determine which glucoside was formed in yeast cells. Yeast strain YZGA515 is incapable of converting DON into 3-ADON due to a deletion of the yeast acetyltransferase gene $AYT1$. This strain was transformed with both the DOGT1 expression vector and a plasmid containing a gene encoding a trichothecene-insensitive mutant ribosomal protein L3 to increase DON tolerance of yeast cells. (Ribosomal protein L3 is the target of trichothecenes.)

Fig. 3. **DOGT1 expression is developmentally regulated and induced by DON and stress response-related compounds.** A, GUS staining of seedlings homozygous for a transcriptional DOGT1 promoter-GUS fusion. Upper row, 3 days after germination (DAG) the expression of the fusion protein is restricted to the vasculature of root and hypocotyl and the meristematic region of the root tip. Middle row, later in development (10 days after germination) staining in the root vasculature diminishes except for regions where lateral roots are formed. Lower row, in aerial parts of adult plants DOGT1 expression is restricted to petals of flowers and abscission zones. B, DON treatment (5 ppm or 16.9 μM for 4 h) of seedlings (14 days after germination) expressing the transcriptional GUS fusion induces expression of the reporter compared with control treatment (Murashige and Skoog medium). Both samples were stained for 2 h. C, semiquantitative reverse transcriptase PCR analysis of induction of expression of DOGT1 following treatment with DON (5 ppm, 16.9 μM), SA (100 μM), JA (50 μM), and ACC (2 μM). UBQ5 (ubiquitin) was used as an internal control. KAN, kanamycin resistance.

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$^7$ G. Adam, unpublished.
After the resulting strain had been incubated with DON, reaching a final concentration of 1000 ppm in the medium, the DON metabolite was extracted from the cells. As shown in Fig. 4C, it was then identified as the expected 3-O-glucopyranosyl-4-deoxynivalenol (Fig. 5A) by HPLC and mass spectrometry. Fig. 4 shows the fragmentation pattern of the synthesized reference substances and the product peak from yeast expressing DOGT1. As expected, the metabolite was not present in the control strain lacking DOGT1 activity (data not shown).

To further verify substrate specificity, a GST-DOGT1 fusion was constructed. This GST-DOGT1 fusion gene conferred DON resistance like wild-type DOGT1 when expressed in yeast. To facilitate in vitro testing, the gene product was expressed in E. coli and affinity-purified. The reaction products generated in vitro during incubation of either the DOGT1 fusion protein or GST with UDP-[14C]glucose and DON were analyzed using TLC. A spot with the same Rp value as the synthesized DON-glucoside was observed in the reaction containing the GST-DOGT1 fusion protein but not in the control (data not shown).

Glucosylation of 4-Deoxynivalenol Greatly Reduces Its Toxicity—To analyze whether the 3-O-glucopyranosyl-4-deoxynivalenol is less phytotoxic than DON, we used a wheat germ extract-coupled in vitro transcription/translation system. As shown in Fig. 5B, 1 μM DON in the reaction mixture inhibited protein translation significantly. Reporter enzyme activity was only 36.8% that of control. 5 μM toxin resulted in only 3.1% luminescence remaining, whereas a 20 μM concentration of the synthesized DON-3-glucoside inhibited luciferase activity only by 8%. These results demonstrate that glucosylation of DON is a detoxification process.

Overexpression of DOGT1 in A. thaliana Increases DON Resistance—Transgenic A. thaliana constitutively expressing DOGT1 under the control of a tandem 35S promoter were generated, and the amount of recombinant protein in transformants was determined by Western blotting utilizing the N-terminal c-Myc epitope tag. Seeds of the homozygous line
**Glucosylation of DON reduces toxicity in vitro and in vivo.** A, structure of the proposed reaction product: DOGT1 catalyzes the transfer of glucose from UDP-glucose to the 3-OH position of DON.

B, comparison of the inhibition of in vitro protein synthesis of wheat ribosomes by DON and DON-3-O-glucoside (DON-3-Gluc) determined using a wheat germ extract-based coupled transcription/translation system. Luminescence was measured and expressed as percent luciferase activity of control samples without toxin.

C, Western blot analysis of A. thaliana lines homozygous for an overexpression construct encoding c-Myc-tagged DOGT1. A schematic drawing of the plant transformation vector is shown below (GENT, gentamycin resistance used as selectable marker; 2x35S, duplicated promoter of cauliflower 35S transcript (see “Experimental Procedures” for details)). Col-0 was used as a negative control. Two lines with high and two lines with low levels of c-Myc-DOGT1 protein are shown. D, seed germination on Murashige and Skoog (MS) medium containing DON (15 ppm or 50.6 μM). Compared with wild-type (Col-0), transgenic Arabidopsis lines expressing high amounts of DOGT1 (e.g. line 1319/2) exhibit enhanced resistance.

**DISCUSSION**

We have cloned a plant UGT that confers resistance to the Fusarium mycotoxin deoxynivalenol by heterologous expression in yeast. Cloning by function, and in particular heterologous expression of plant UGT genes in yeast, is a valuable complementary approach to the widely used E. coli expression systems. This is especially true when the respective chemicals have targets in eukaryotes only. One problem, however, is that wild-type yeast cells are frequently “impermeable” to the substances of interest. Inactivating several ATP-binding cassette transporters in our host strain was a prerequisite for selection of cDNAs conferring resistance to Fusarium toxins. In the case of trichothecenes this approach allowed phenotypic detection of detoxification activity by a simple plate assay. In principle, this approach could be adopted for many other substances.

**Biotechnological Relevance—Fusarium** diseases of wheat and barley are of high economic significance for countries around the world. The United States for instance has been severely hit by Fusarium epidemics in the last decade. Direct losses to wheat producers in the United States due to Fusarium head blight have been estimated to average about $280 million annually, and over the period 1998–2000 the combined economic losses for small grain cereals were estimated at $2.7 billion (41). Deoxynivalenol contamination of large portions of a harvest can lead to high human intake of the mycotoxin. Children are the population group most at risk to exceed the tolerable daily intake level for DON. In the problematic year 1998 80% of 1-year-old children in the Netherlands exceeded the tolerable daily intake (8). Thus, methods to prevent and/or treat Fusarium diseases in grains are extremely important. This high importance of Fusarium diseases clearly justifies research on mycotoxin inactivation, although it is very likely that pathogen resistance is a polygenic trait in crop plants, and toxin resistance is just one of its components.

The production of trichothecenes is the only virulence factor of F. graminearum (Gibberella zeae) for which the mode of action is known. It is therefore a prime target for biotechnological approaches to combat the fungus. Other genes required for full virulence have been reported (42–44). Yet mutations in mitogen-activated protein kinases are pleiotropic, affecting not only virulence but also conidiation, perithecia formation, vegetative growth, and mycotoxin production. In the case of the recently identified CPS1 gene (44), it is unclear whether the hypothetical adenylate-forming enzyme encoded by the gene is involved in biosynthesis of an unidentified metabolite or necessary for some unknown aspect of stress tolerance, in particular in plants mounting a defense response.

Our finding that overexpression of DOGT1 leads to increased deoxynivalenol resistance in transgenic Arabidopsis is signifi-
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It may open new possibilities for biotechnological approaches aiming to antagonize the fungal virulence factor. So far, such attempts rely on a *Fusarium* acetyltansferase (45) that converts DON into 3-ADON, which is ~2-fold less toxic to laboratory animals and nearly as toxic as DON to wheat (6).

The phenotype of our transformed yeast and laboratory animals and nearly as toxic as DON to wheat (6). /H11011

The analysis of expression of *DOGT1* showed elevated mRNA levels in response to SA, JA, and the ethylene precursor ACC. The inducibility of gene expression by SA, JA, or ethylene is considered to be indicative for a possible role of the up-regulated gene product in plant stress or defense responses (52).

Using analysis of mRNA levels and a GUS-reporter construct we were able to show that *DOGT1* transcription in wild-type *A. thaliana* is developmentally regulated and is rapidly and significantly induced in response to DON exposure. It would be interesting to clarify whether the inducibility is compound-specific or represents a general response to protein biosynthesis inhibitors. The “ribotoxic stress response” is currently being actively investigated by researchers in human cell systems (53). In these cells, the myotoxic induces expression of cyclooxygenase-2, a key enzyme in the synthesis of inflammatory response mediators, via mitogen-activated protein kinase-mediated signaling. The DON-inducible expression of *DOGT1* is an attractive starting point for investigating whether similar mechanisms exist in plants.

In summary, we propose that members of the huge gene family of UGTs could play an important role in plant-pathogen interaction by participating in detoxification of metabolites produced by microbes to increase their virulence on hosts. The selective pressure to escape such glycosylation reactions may be a driving force in evolution of microbial biosynthetic reactions leading to a wide spectrum of toxin structures as observed for trichothecenes.

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