Functional Characterization of Two Clusters of *Brachypodium distachyon* UDP-Glycosyltransferases Encoding Putative Deoxynivalenol Detoxification Genes

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Plant small-molecule UDP-glycosyltransferases (UGT) glycosylate a vast number of endogenous substances but also act in detoxification of metabolites produced by plant-pathogenic microorganisms. The ability to inactivate the *Fusarium graminearum* mycotoxin deoxynivalenol (DON) into DON-3-O-glucoside is crucial for resistance of cereals. We analyzed the UGT gene family of the monocot model species *Brachypodium distachyon* and functionally characterized two gene clusters containing putative orthologs of previously identified DON-detoxification genes from *Arabidopsis thaliana* and barley. Analysis of transcription showed that UGT encoded in both clusters are highly inducible by DON and expressed at much higher levels upon infection with a wild-type DON-producing *F. graminearum* strain compared with infection with a mutant deficient in DON production. Expression of these genes in a toxin-sensitive strain of *Saccharomyces cerevisiae* revealed that only two *B. distachyon* UGT encoded by members of a cluster of six genes homologous to the DON-inactivating barley HvUGT13248 were able to convert DON into DON-3-O-glucoside. Also, a single copy gene from *Sorghum bicolor* orthologous to this cluster and one of three putative orthologs of rice exhibit this ability. Seemingly, the UGT genes undergo rapid evolution and changes in copy number, making it difficult to identify orthologs with conserved substrate specificity.

The fungal hemibiotrophic pathogen *Fusarium graminearum* and closely related species (Sarver et al. 2011) are able to infect a broad range of crop plants (Goswami and Kistler 2004; Kazan et al. 2012), most importantly wheat (*Triticum aestivum*) and

barley (*Hordeum vulgare*) but also maize (*Zea mays*). In addition, dicotyledonous hosts ranging from potato (*Solanum tuberosum*) (Delgado et al. 2010) to sugar beet (*Beta vulgaris*) (Christ et al. 2011) and *Arabidopsis thaliana* (Urban et al. 2002) can also be infected. Presumably, the fungus can suppress resistance in a broad range of host plants by either effector proteins or small molecule effectors. Little is known about the role of the numerous potential secondary metabolites that the fungus seems to be able to produce, as revealed by the genome sequence (Cuomo et al. 2007). According to the *F. graminearum* database (Wong et al. 2011), it contains 15 predicted genes encoding polyketide synthases, 19 nonribosomal peptide synthetases, and 21 putative genes involved in biosynthesis of terpenoids (17 terpenoid synthases [IPR008949] and four terpenoid cyclases/protein prenyltransferases [IPR008930]). For only a small fraction of these genes, corresponding metabolites are known (Hansen et al. 2012). Deoxynivalenol (DON) is a sesquiterpene and prominent member of the class of trichothecene mycotoxins, which inhibit eukaryotic protein synthesis (Cundliffe and Davies 1977). DON attracted broad scientific interest because it is also highly toxic to humans and animals consuming *F. graminearum*-infected and DON-contaminated grain (European Commission 1999; Pestka 2010). To protect consumers, harsh maximum tolerated levels of DON in cereals and commodities intended for human consumption were enacted in Europe (European Commission 2006). The biosynthetic pathway for DON production has been, for the most part, elucidated (McCormick et al. 2011).

Disruption of the *TRI5* gene encoding trichodiene synthase, the first dedicated step in DON biosynthesis, results in reduced virulence of the fungal pathogen on wheat (Proctor et al. 1995). The *tri5* mutants can still infect but are challenged in their ability to spread from the infection site to other spikelets in wheat (Bai et al. 2002). It has been demonstrated (Jansen et al. 2005) that, in a susceptible wheat cultivar, spreading of a *tri5* mutant to neighboring spikelets was blocked by cell wall thickenings in the rachis nodes, and that this process was suppressed during infection with the toxin producing wild-type *F. graminearum*. Resistance to *F. graminearum* head blight in wheat and other cereals is a complex, polygenically inherited trait (Buerstmayr et al. 2009). A major quantitative trait locus in wheat, *Qfhs.ndsu-3BS* (containing the hypothetical *F. gra*-

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minearum resistance gene *Fhb1*) restricts the spread of disease symptoms (Buerstmayr et al. 2002; Waldron et al. 1999). In a wheat population segregating for *Fhb1*, lines containing the *Fhb1* resistance allele were shown to efficiently conjugate DON to the less toxic DON-3-O-glucoside (D3G) (Lemmens et al. 2005). Recently, it has been shown by comparison of near-isogenic lines differing in *Fhb1* that the rachis of the resistant line contained much higher levels of various phenylpropanoids and, in particular, phenylpropanoid amides (Gunnaiah et al. 2012). These authors did not find significantly different levels of DON and D3G in the infected spikelet and, therefore, repudiated the hypothesis that *Fhb1* is based on increased glycosylation capacity leading to increased DON resistance. Yet, this is difficult to reconcile with results of studies using pure toxin (Horevay et al. 2012; Lemmens et al. 2005). The seemingly conflicting results can be explained by the hypothesis that, in the heavily inoculated spikelet, resistance is overwhelmed but that DON diffusing ahead of the fungus induces UDP-glycosyltransferases (UGT) in neighboring tissue in the resistant line, eventually antagonizing the virulence factor. Potentially, there could also be differences between cultivars in the further metabolism of D3G to other metabolites, such as DON-malonylglucoside and the recently described DON-di/tri/tetra-glucosides (Zachariasova et al. 2012).

The first UGT gene (*DOG1*, *AtUGT73C5*) encoding an enzyme capable of DON detoxification was identified by selection of an *A. thaliana* cDNA clone that rescued a toxin-sensitive yeast strain on DON-containing media (Poppenberger et al. 2003).

UGT are encoded by a very large gene family, with well over 100 members in different plant species (Bowles et al. 2005). Identification of specific UGT from crop plants such as wheat, which conjugates DON to D3G, turned out to be very difficult (Schweiger et al. 2011). Numerous candidate UGT genes and gene fragments that are induced during *F. graminearum* infection were identified in different studies (Desmond et al. 2008; Hill-Ambroz et al. 2006; Lulin et al. 2010; Steiner et al. 2009; Walter et al. 2008). Transcriptomic studies in barley have led to the identification of candidate genes, which are induced by DON application and by infection with a DON-producing wild-type *F. graminearum* but not with a DON-deficient *tri5* mutant (Boddu et al. 2006, 2007; Gardiner et al. 2010). Nine of these barley UGT were tested by heterologous expression in a DON-sensitive yeast strain but only one gene, designated *HvUGT13248*, could be validated and was able to confer DON resistance via the conjugation of DON to D3G (Schweiger et al. 2010; M. P. Kovalsky Paris, *unpublished data*). Theoretically, the same resistance mechanism should operate in different crop plants and contribute to the complex trait *F. graminearum* resistance by antagonizing the virulence factor DON. Orthologs of the genes identified in *A. thaliana* or barley are obvious candidates to play such a role. Because the genomes of wheat and barley are not sequenced yet, we de-

cided to analyze the architecture of the UGT gene family from the closely related monocot model plant *Brachypodium distachyon*, for which the genome sequence is available (International Brachypodium Initiative 2010). The goal of our study was to functionally test genes with the highest similarity to the previously identified *A. thaliana* and barley UGT encoding DON detoxification enzymes (*AtUGT73C5* and *HvUGT13248*). We also tested putative orthologs of candidates from the more distantly related and sequenced crop plants rice (*Oryza sativa*) and sorghum (*Sorghum bicolor*). Our results indicate that translational research with the goal to identify corresponding DON detoxification genes across a range of crop plants is nontrivial due to the rapid changes in copy number of clusters of UGT and changes in substrate specificity of highly similar genes.

RESULTS

Architecture of the UGT gene family of *B. distachyon*.

Small-molecule-conjugating UGT, also known as family 1 UGT, are a very large gene family in plants that has been characterized phylogenetically and, in part, functionally in *A. thaliana* (Paquette et al. 2003; Ross et al. 2001), and to a limited extent in other species (Gachon et al. 2005). Information on UGT of crop species is generally scarce. *B. distachyon* is the closest sequenced relative of wheat and barley. Therefore, we decided to characterize the *B. distachyon* small-molecule UGT gene family. A hallmark of these enzymes is a motif called PSPG-box (Gachon et al. 2005; Vogt and Jones 2000), which is part of the region interacting with the UDP-sugar co-substrate. Using the sequence WAPQVEVLAHPAVGCFVTHCGWNSTLESISAGVPMVAWPPFFADQ (Vogt and Jones 2000) as a query with the BlastP algorithm (E value < e^{-10}), 144 putative UGT genes were identified in the *Brachypodium* genome database (gene model version 1.2) hosted by the Munich Information Center for Protein Sequences (MIPS). A query for the corresponding entry in the Pfam protein family database (PF00201, UDP-glucuronosyl, and UGT) yielded 166 gene models. Searching for the InterPro domain motif IPR002213 (UDP-glucuronosyl/UGT) identified 178 gene models.

The *B. distachyon* UGT gene loci predicted by InterPro are dispersed throughout all five chromosomes (Supplementary Fig. S1) but frequently form clusters of up to seven genes. We denoted 13 gene models as probable pseudogenes. Compared with other genes they seem to encode N- or C-terminally truncated and, therefore, nonfunctional proteins (Supplementary Table S1). In contrast, Bradi2g34500.1, spanning a predicted 2,844-bp open reading frame (ORF), is considered to be a pseudogene due to its unusually large size. The remaining 164 UGT genes range in ORF length between 1,152 and 1,836 bp. The average gene length of 1,450 bp is consistent with the average UGT ORF length calculated for barley (1,452 bp) from a set of 76 UGT coding sequence that have been made publicly available with a library of full-length cDNAs (Matsumoto et al. 2011). For four genes, at least one alternative gene model is predicted, differing in the presence or absence of a hypothetical intron. We chose the versions with the highest confidence class for further analysis (Bradi1g27290.2, Bradi2g49057.1, Bradi2g47420.1, and Bradi3g07997.4). In summary, by subtracting 14 pseudogenes and five hypothetical alternative splicing forms of four different genes from the identified 178 predicted gene models, we concluded that the *B. distachyon* genome contains 159 genes encoding potentially functional UGT.

The *B. distachyon* UGT amino acid sequences were submitted to the UDP-Glucuronosyltransferase Nomenclature Committee, which provided systematic names (Supplementary Table S2) following the published guidelines (Mackenzie et al. 1997).

Table 1. Systematic names of UDP-glycosyltransferase gene loci characterized in this study

Locus name	Clade	Systematic name
Bradi2g04700.1	D1	UGT99B5
Bradi2g04720.1	D1	UGT98B5
Bradi2g04747.1	D1	UGT98B6
Bradi2g04760.1	D1	UGT98B7
Bradi5g02780.1	L1	UGT74J7
Bradi5g03300.1	L1	UGT74J9
Bradi5g03370.1	L1	UGT74J12
Bradi5g03380.1	L1	UGT74J8
Bradi5g03390.1	L1	UGT74J10
Bradi5g03400.1	L1	UGT74J11

For UGT described in more detail, the systematic names are given in Table 1. To assess the evolutionary relationship between *B. distachyon* UGT and the already established UGT-phylogeny of *A. thaliana*, the 159 *B. distachyon* UGT amino acid sequences were compared with 122 *A. thaliana* UGT amino acid sequences listed currently in the *A. thaliana* cytochrome 450 and UGT database in a combined multiple alignment and phylogenetic analysis (ClustalW2, neighbor-joining, 1,000 bootstrap iterations). The results are shown in Figure 1. In *B. distachyon*, we identified 24 clades that, based on this combined phylogeny, share ancestral genes with 22 orthologous groups in *A. thaliana*. Within most clades, UGT from *A. thaliana* and *B. distachyon* form distinct groups, suggesting

that these have evolved separately in both species. The *B. distachyon* clades were denominated after the nomenclature introduced for *A. thaliana* by Ross and associates (2001). Subgroups (e.g., clades L1 and L2) were added when *B. distachyon* clades were more differentiated than the original *A. thaliana* clades.

Most *B. distachyon* UGT genes contain either no intron (96 gene models) or only one (50 models). Eleven gene models harbor two or three introns and only Bradi2g56102.1 and Bradi3g07997.4 exhibit a more complex intron/exon structure, with 13 or 14 introns, respectively. These genes are most similar to sterol-UGT, which also contain an exceptionally high number of introns and splice variants in other species, including *A. thaliana* (DeBolt et al. 2009).

Fig. 1. Combined phylogenetic analysis of *Brachypodium distachyon* and *Arabidopsis thaliana* UDP-glycosyltransferase (UGT) amino acid sequences. Evolutionary related clades are colored in alternating blue and red. *Arabidopsis* UGT are shaded in light colors and *B. distachyon* UGT in dark colors. Bootstrap (1,000×) confidence values lower than 65 are given in gray and values lower than 40 in dashed gray lines. Cluster denomination for *B. distachyon* has been adapted from *Arabidopsis* (Ross et al. 2001). Individual subgroups have been further separated (e.g., A1 and A2). Deoxynivalenol (DON)-responsive genes reported by Schweiger and associates (2010) have been integrated into the phylogeny (no colored background, clades D1, D2, and L1).

Excluding the sterol-UGT, we identified a minimum of 23 intron insertion events and five intron losses by comparing the distribution of introns with the phylogenetic analysis based on the amino acid sequences (Supplementary Fig. S2). Although many introns are specific for single genes or are only shared

by few paralogous genes, one intron was found conserved across 36 genes in seven clades (F, K, J, G, H, N, and I): all but one of these introns share the same intron phase (the position of the intron within a codon) and all introns reside at approximately the same position (478 to 562 bp), suggesting a

Fig. 2. Induction of *Brachypodium distachyon* UDP-glycosyltransferase (UGT) genes by either **A**, *Fusarium graminearum* wild-type strain PH-1 and the $\Delta tri5$ -mutant MU102, which has lost the ability to produce deoxynivalenol (DON); or **B**, treatment with DON.

single insertion event in an ancestral monocot gene. Orthologous genes to these *B. distachyon* UGT genes in *A. thaliana* also harbor a conserved intron but at a different position (at approximately 279 bp) (Paquette et al. 2003). The *B. distachyon* UGT genes of clade L1 located on chromosomes 4 and 5 share an ancestral intron (starting approximately 680 bp downstream of the ATG) and apparently a second, more recent insertion event occurred in a subclade on chromosome 5. All other introns are, in most cases, present in only one single gene. A detailed comparison of intron positions and phases of *B. distachyon* UGT is given in Supplementary Table S3.

Genes encoding enzymes conferring the ability to inactivate toxins may be under positive selection to allow adaptation to new pathogens. The sesquiterpenoid toxins of the class of trichothecenes, which are produced mainly by plant-pathogenic fungi, are highly diverse; at least 217 naturally occurring compounds have been described (Grovey 2007). *F. graminearum*, for instance, may produce either DON or the structurally similar nivalenol, which has one additional hydroxyl group at carbon four. Despite this similarity, the *A. thaliana* UGT73C5 is unable to inactivate nivalenol. The C-terminal domain of UGT required for binding the co-substrate UDP-sugar (UDP-glucose in most cases) might evolve at a much slower pace than N-terminal domains responsible for specific interaction with the substrate. To investigate whether there is evidence for selective pressure in UGT gene families, we calculated ω , the ratio of nonsynonymous (dN) to synonymous (dS) nucleotide changes for alignments of individual domains and also single-codon sites, taking into account potential false-positive interferences as a result of recombination. None of the groups of UGT domains showed a dN/dS ratio higher than 1—the commonly used threshold for positive selection. N termini (average $\omega = 0.242$) and C termini (average $\omega = 0.245$) exhibited a similarly conserved pattern, which was also true for the average ω calculated for the conserved signature PSPG motif ($\omega = 0.16$). The only exception was clade L2, for which ω of the N-terminal domain (0.54) was approximately 2.6-fold higher than ω of the C-terminal domain. However, no single-codon sites under positive selection have been identified for L2. In contrast, clades B, H, E2, and especially D2 harbor up to seven sites under positive selection (Supplementary Table S4). Neither clade L1, which contains genes most similar to *HvUGT13248*, nor clade D1, containing genes most similar to *AtUGT73C5* (*DOG1*), showed signs of diversifying selection.

***B. distachyon* homologs to the *A. thaliana* DON-UGT *AtUGT73C5* (*DOG1*) are responsive to DON but their products do not inactivate the toxin.**

The close relatedness of *B. distachyon* to cereal crop species should allow us to gain insights into the evolutionary history of candidate genes based on the phylogeny. We identified *B. distachyon* orthologous gene clusters to the *F. graminearum*/DON-responsive UGT from barley and wheat that have been functionally characterized (Schweiger et al. 2010) by adding the protein sequences encoded by these genes to the combined *A. thaliana*/*B. distachyon* UGT phylogeny (Fig. 1; *HvUGT5876*, *HvUGT13248*, *HvUGT19290*, *HvUGT14077*, and *TaUGT3*). In all cases, the cereal UGT are interspersed within *B. distachyon* clades, reflecting the expected more distant relationship to the dicot *A. thaliana*.

The gene products of *TaUGT3* and *HvUGT5876*, which are both induced by DON (Gardiner et al. 2010; Lulin et al. 2010) but lack the ability to conjugate the toxin (Schweiger et al., 2010), are closely related to *B. distachyon* clade D1 and, thus, are putative orthologs. The closest homologous group in *A. thaliana* is cluster 73C (with average sequence identities between 36.4 and 42%) containing the DON-inducible and DON-

Fig. 3. A, UDP-glucosyltransferase (UGT) activity catalyzes the transfer of glucose from UDP-glucose to the 3-OH position of deoxynivalenol (DON), forming DON-3-O-glucoside. **B**, Spotting of independent yeast transformants expressing *Brachypodium distachyon* UGT homologous to the DON-inactivating *AtUGT73C5* (3 and 4), and UGT syntenous to the *B. distachyon* UGT family L1 of sorghum (5) and rice (6–8) on plates containing increasing concentrations of DON. Strains harboring the empty plasmid (1) or expressing the previously reported UGT from barley with the ability to inactivate DON (2, *HvUGT13248*) were used as controls.

inactivating UGT AtUGT73C4 and AtUGT73C5 (Poppenberger et al. 2003; Schweiger et al. 2010). Clade D1 in *B. distachyon* comprises four genes: Bradi2g04720.1, Bradi2g04747.1, Bradi2g04760.1, and Bradi2g04700.1 (Table 1). These genes are located on chromosome 2 and share a common intron. With the exception of the more distantly related Bradi2g04700.1 (maximum sequence identity 36.1%), the UGT show a high level of amino acid sequence similarity (at least 80% identity, ClustalW) within the *B. distachyon* group and also with the barley and wheat UGT HvUGT5876 and TaUGT3. Yet, Bradi2g04747.1 seems to be a nonfunctional gene. The gene model predicts an additional intron to maintain the ORF, leading to a shorter UGT, which lacks an internal stretch of 56 amino acids compared with other UGT in the family. Therefore, we focused on the two remaining UGT most similar to AtUGT73C5, HvUGT5876 and TaUGT3. We performed quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) for Bradi2g04720.1 and Bradi2g04760.1 to test whether these genes showed a response to *F. graminearum* stress or DON application similar to that of the reported barley gene and the wheat gene, which has only been tested regarding its inducibility by DON. We inoculated single spikes of *B. distachyon* ecotype Bd21 plants with *F. graminearum* strains PH-1 (wild type, DON-producer) and MU102, a $\Delta tri5$ mutant lacking the ability to produce DON (Cuzick et al. 2008). Both UGT genes were highly expressed in response to either *F. graminearum* strain but inoculation with the DON-producing wild-type strain led to a much higher transcript accumulation (Fig. 2A). We found transcripts corresponding to Bradi2g04720.1 approximately 20-fold and Bradi2g04760.1 approximately 52-fold more abundant at 96 h after inoculation (hai) in samples treated with PH-1, compared with samples inoculated with the DON-deficient mutant MU102.

In a separate experiment, we treated plants with 2 μ g of DON per spikelet. This led to highly elevated transcript levels compared with mock-treated samples. At 12 hai, Bradi2g04720.1 was 700-fold more abundant and Bradi2g04760.1 more than 80-fold (Fig. 2B).

In an effort to test whether these *Brachypodium* genes would be able to form the detoxification product D3G (Fig. 3A), we cloned and expressed the cDNAs of both genes in the toxin-sensitive *Saccharomyces cerevisiae* strain YZGA515. The expression cassette of the yeast plasmid contains a strong constitutive *ADHI* promoter with an N-terminal c-Myc epitope tag in frame with the cDNA insert (Schweiger et al. 2010). The DON-sensitive yeast strain YZGA515 (relevant genotype: *pdr5*

pdr10 pdr15 ayt1) was transformed and biological replicates were spotted on yeast peptone dextrose (YPD) plates containing increasing amounts of DON. Presence of both UGT gene products could be confirmed by immunodetection of the c-Myc tag (Supplementary Fig. S3). Neither of the strains developed a resistant phenotype even on low concentrations of DON as compared with the control strain harboring only the empty plasmid (Fig. 3B).

Two *B. distachyon* UGT similar to HvUGT13248 inactivate DON by formation of D3G.

The DON-inactivating barley UGT HvUGT13248 clusters with a family of six UGT within *B. distachyon* clade L1 (Fig. 1). This gene family comprises Bradi5g02780.1, Bradi5g03300.1, Bradi5g03370.1, Bradi5g03380.1, Bradi5g03390.1, and Bradi5g03400.1 (Table 1). The genes form a cluster on chromosome 5 with a core region of 60 kb, with the exception of Bradi5g03400.1, which is located approximately 800 kb upstream of this region (Fig. 4). Members of the gene cluster share 51.6 to 83.3% amino-acid sequence identity. The gene products with the highest similarity to HvUGT13248 are Bradi5g03300.1 and Bradi5g03390.1, with 71.6 and 71.8% amino acid sequence identity, respectively (Fig. 5A). All UGT genes in the cluster contain two introns separating an N-terminal 684-bp exon from two C-terminal exons of 385- and 334-bp average lengths. The annotation for Bradi5g03400.1 predicts an additional 179-bp exon at the N terminus of the protein and also a 5' truncated exon 3, when compared with the respective exons 2 of the other family members. The missing homologous fragment of 219 bp in length is found in the 5' intronic sequence. Available expressed sequence tag data are inconsistent as to the reliability of this gene model. However, no in-frame splice site consensus sequences exist in the region that would support an alternative gene model. The cluster furthermore harbors two UGT pseudogenes (Bradi5g03310.1 and Bradi5g03320.1), which are remnants of the N- and C-terminal part of a UGT gene that has been disrupted by an insertion event.

In qRT-PCR experiments, all six UGT showed a moderate to high response to DON and were more responsive to wild-type PH-1 than to the DON-minus *tri5* mutant strain MU102 (Fig. 2A and B). The lowest expression changes were observed for Bradi5g03400.1. As shown before for the *AtUGT73C5* orthologous BdUGT genes, the highest transcript abundances in response to *F. graminearum* infection for all investigated genes were observed 96 hai. Bradi5g03300.1 was induced 1,100-fold,

Fig. 4. Comparative genomic organization of the deoxynivalenol (DON) or *Fusarium graminearum*-responsive *Brachypodium distachyon* UDP-glycosyltransferase (UGT) gene cluster with the syntenous gene family in rice. Top row illustrates a region of chromosome 5 of *B. distachyon* harboring UGT genes Bradi5g02780.1, Bradi5g03300.1, Bradi5g03370.1, Bradi5g03380.1, Bradi5g03390.1, and Bradi5g03400.1. The two putative pseudogenes Bradi5g03310.1 and Bradi5g03320.1 are also indicated. Bottom row represents the syntenous gene region on chromosome 4 of rice, including five UGT gene models. The putative ortholog of Bradi5g02780.1 is NM_001058780.1 (78.0% identity). Bradi5g03380.1 is related to NM_001058778.1 (74.7%), and Bradi5g03390.1 to NM_001058779.1 (68.9%, ClustalW protein alignment). All annotated UGT gene models are denominated and represented in black. Gene models encoding for genes other than UGT are shaded in gray. Bars linking single gene loci across species indicate the putative orthologous pairs.

Bradi5g03370.1 150-fold, and Bradi5g03380.1 560-fold. Dramatic changes were also observed after DON treatment, with the highest ratios between DON and mock treatment at 8 or 12 hai. Bradi5g02780.1 had the highest increase in transcript abundance at the earlier time point (3 hai, 28-fold induction).

For functional testing by heterologous expression in yeast, the coding sequences of all six paralogous genes in clade L1 were recoded for optimal expression in yeast, custom synthesized, and transformed into strain YZGA515. The spotting assays showed that two of the six UGT tested conferred higher resistance to DON compared with strains containing only the empty vector control (Fig. 5B). Transformants expressing Bradi5g03300.1 conferred a high level of resistance, similar to that of the positive controls expressing *HvUGT13248* or *DOG1*, whereas transformants expressing Bradi5g02780.1 could only grow on plates containing DON at 40 mg/liter or less. Again, presence of UGT proteins was confirmed by immunodetection.

To confirm that resistance is caused by the ability of both *B. distachyon* UGT to convert DON into D3G, liquid cultures of strains expressing either gene or *HvUGT13248* as a positive control were incubated with DON at 500 mg/liter. Samples of culture supernatants taken at different time points were examined using high-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS), as previously described (Berthiller et al. 2009). Strains expressing Bradi5g03300.1 and *HvUGT13248* accumulated intracellular D3G at similar amounts (average of 701 and 641 µg/liter, respectively, after 24 h of incubation) (Supplementary Fig. S4). The majority of the D3G was already formed after 8 h of incubation. Transformants expressing Bradi5g02780.1 were also able to produce D3G, yet the glucoside was only detected after 16 and 24 h of incubation and the D3G levels were lower (535 µg/liter in average) at the end point of 24 h of incubation.

Synteny of the *B. distachyon* UGT cluster to rice and sorghum.

To estimate the extent to which this gene family has evolved differently in other related grass species, we searched for homologous loci to the six *B. distachyon* genes using CrowsNest—a comparative mapviewer hosted by MIPS. The analysis is based on transcript annotation of rice and *B. distachyon* derived from the MIPS PlantsDB. We identified a syntenic region comprising a cluster of five UGT genes on chromosome 4 of rice (Fig. 4). The core cluster is formed by four genes, three of which are most likely functional (NM_001058778.1, Os04g0206500; NM_001058779.1, Os04g0206600; and NM_001058780.1, Os04g0206700) plus a 374-amino-acid gene fragment (NM_001186865.1, Os04g0206450), which is C-terminally truncated and devoid of an initiation codon. Meanwhile, a hypothetical N-terminally truncated pseudogene now considered to be a genome assembly artifact, (NM_001058775.1, Os04g0205900) is removed from most databases.

The three rice UGT genes (NM_001058778.1, NM_001058779.1, and NM_001058780.1) exhibited a higher similarity to the *B. distachyon* homologs than to any of the rice paralogs, suggesting that these pairs form true orthologs. A similar analysis for *Sorghum bicolor* identified XM_002447461.1 (Sb06g002180) as the only putative orthologous gene. The sorghum gene as well as the three rice genes (NM_001058778.1, NM_001058779.1, and NM_001058780.1) were custom synthesized for expression in yeast and functionally tested with the yeast-spotting assay. We found that only one of the rice genes, NM_001058779.1 (short designation OsUGT79) and the sorghum gene conferred resistance to DON at a level similar to that of the barley *HvUGT13248* gene (Fig. 3B).

DISCUSSION

In this study, we have characterized the large gene family of *Brachypodium* small-molecule conjugating glycosyltransferases using bioinformatic tools. According to our analysis, the

Fig. 5. A, Phylogenetic relationship of the deoxynivalenol (DON)-responsive *Brachypodium distachyon* UDP-glycosyltransferase (UGT) genes of clade L1 located on chromosome 5 (ClustalW protein alignment, neighbor-joining, 1,000× bootstrap). DON-inactivating UGT are underlined. **B**, Spotting of two individual yeast transformants expressing either of these genes or controls from barley (*HvUGT13248*), *Arabidopsis* (*AtUGT73C5*), or the empty vector on plates containing the indicated concentration of DON.

178 predicted gene models constituting this gene family include 159 genes with the capacity to encode proteins of the typical size characteristic for the known functional UGT. We excluded Bradi2g04747.1 from the functional analysis because it seemed to contain an internal deletion resulting in a protein, which is shorter than the other cluster members, but retained Bradi5g03400.1, although the gene model seemed suspicious because parts of the predicted intron contained sequences with similarity to coding sequences in closely related genes. Phylogenetic analyses identified 24 clades, 22 of which share a common ancestor with those previously identified in *A. thaliana*. Within common clades, sequences from the two species are well separated, revealing that sequence divergence occurred before speciation. We identified two *B. distachyon* clades that contain homologs of the DON-inactivating UGT from the dicot model plant *A. thaliana* (Poppenberger et al. 2003) or barley (Schweiger et al. 2010), respectively. All candidate genes tested showed in planta inducibility by DON and higher induction upon infection with a DON-producing wild-type *F. graminearum* than infection with a *tri5* mutant.

Despite the responsiveness to DON (Fig. 2), only two of the eight tested genes conferred DON resistance when tested by heterologous expression in yeast. The finding that none of the *B. distachyon* genes in clade D1 is able to detoxify DON is in line with the recent experiments performed with wheat and barley orthologs (Schweiger et al. 2010) as well as the corresponding rice candidate genes (Schweiger et al. 2011), all of which were unable to confer DON resistance. The *A. thaliana* DON-UGT AtUGT73C5 is the closest homolog to these genes, yet shares only approximately 40% sequence identity. A possible explanation for this observation is that ancestral monocot UGT of clade D1 have lost the ability to inactivate the toxin—a role that was taken over by a member of clade L1 and possibly other untested genes. Alternatively, these results could reflect independent evolution of the ability to detoxify DON in different plant species.

Sequence diversification in gene clusters maintaining DON inducibility but leading to loss of the ability to glycosylate DON in different members is obviously occurring: in the cluster carrying the six L1-type *B. distachyon* UGT on chromosome 5, only two confer DON resistance but all show DON inducibility. Yet, these genes are not simply alleles with a loss of function. Bradi5g02780.1, for instance, which confers resistance only to low levels of DON, results in high-level resistance to a different trichothecene toxin when expressed in yeast (W. Schweiger, unpublished data). Similarly, one of the DON-induced UGT from barley that is inactive on DON (Schweiger et al. 2010) can glycosylate another *F. graminearum* metabolite, which is structurally unrelated to trichothecenes (M. P. Kovalsky Paris, unpublished data). DON-induced transcription of genes lacking DON-detoxification capability might have a biological purpose, by priming defense against other unconsidered or unknown metabolites. It may also be biologically relevant that the *B. distachyon* gene Bradi5g03300.1 showing the highest and most rapid induction upon challenge with the fungus in this study confers resistance against the highest DON concentrations. In *A. thaliana*, genes clustering in clade L1, such as AtUGT74B1 and 74E2, have no known effect on DON but do have functions related to auxin-conjugation (indole-3-acetic acid and indole-3-butyric acid) (Grubb et al. 2004; Tognetti et al. 2010). Overexpression of members of the neighboring clade L2 also affects auxin homeostasis (AtUGT84B1) (Jackson et al. 2002). These data further reflect how substrate specificity of UGT is difficult to predict based only on phylogenetic-based homology. It remains a matter of speculation as to why the UGT detoxifying DON have very low basal expression levels. It is obvious that simultaneous overexpression of

many UGT in the same cell would be counterproductive, mainly because they would compete for the co-substrate which, in most cases of plant glycosyltransferases, is UDP-glucose. Due to the broad substrate-specificity of UGT, they would potentially conjugate other endogenous substrates: constitutive overexpression of *AtUGT73C5* leads to glycosylation of brassinosteroids and, consequently, to dwarfing (Poppenberger et al. 2005). However, this is not the case for *A. thaliana* plants overexpressing *HvUGT13248*, which show high-level DON resistance without displaying unwanted side effects regarding plant anatomy (Shin et al. 2012). Nevertheless, tissue-specific and developmental regulation of expression might be crucial to diminish interference with endogenous metabolites, especially growth hormones and defense signal molecules such as salicylic acid (e.g., AtUGT74F1 in clade L1) (Song et al. 2008). It is conceivable that gene products with an original role in plant secondary metabolism have some side activity providing a low level of resistance against a new microbial toxin. The simplest way to increase the detoxification capability would be amplification of such a gene, followed by sequence diversification and functional specialization of cluster members.

In analogy to the leucine-rich repeat (LRR) disease-resistance gene clusters, which show diversifying selection in the domain, presumably interacting with the rapidly changing effector protein repertoire of pathogens (Meyers et al. 1998), one would expect that diversifying selection might also be detected in UGT that interact with “small-molecule effectors” of plant pathogens. In the case of UGT, the C-terminal part containing the sugar-co-substrate binding domain is more conserved, whereas the N-terminal part is mainly responsible for substrate binding, as revealed by hybrid proteins (Osmani et al. 2009; Schweiger et al. 2010). If UGT gene cluster members are under diversifying selection, one would expect to observe differences in the rate of synonymous to nonsynonymous changes. From our results, we conclude that this scenario is rather unlikely in some clusters. No evidence for diversifying selection was found in the L1 gene cluster containing the DON-detoxification genes. Therefore, the interaction of grasses with trichothecenes seems to be a case of trench warfare (Friedman and Baker 2007; Theis and Stahl 2004), predating speciation in cereals and *F. graminearum* (Ward et al. 2002), rather than of a rapid arms race with massive changes in the toxin spectrum.

Like the nucleotide-binding site LRR disease-resistance genes, the UGT seem to undergo rapid changes in copy number. In the case of the genes most similar to *HvUGT13248*, we found six homologous candidate genes in *B. distachyon* and three genes and one gene in the corresponding syntenic regions of rice and *S. bicolor*, respectively. Gene copy-number variation has also been observed for other grass gene families such as the prolamine genes, where evidence for gene birth by insertion of copies in other chromosomal regions, formation of clusters by tandem duplications, and gene death by unequal crossing over and truncation by insertion events were also reported (Xu et al. 2012). Such deletions of genes may be without consequences as long as functional copies remain in the genome. Therefore, which of the paralogous genes of a cluster remains active (or loses this function and gains others) may be largely unpredictable, especially in genomes of allopolyploid plants such as hexaploid wheat. Large gene families also seem to be prone to genome asymmetries, where orthologous genes are located in different chromosomal positions in different cultivars (Ding et al. 2007). It has been proposed that the region spanning the *Fhb1* locus should contain a UGT-inactivating DON (or a regulator) (Lemmens et al. 2005). No small-molecule UGT was found in the sequenced region of the susceptible ‘Chinese Spring’ (Choulet et al. 2010). It remains

to be determined whether the corresponding region in resistant cultivars contains one or several UGT genes.

As shown for the animal chemosensory receptors, the number of genes encoding functional receptors and the number of pseudogenes vary enormously between genomes of different animals. Genomic drift generating copy-number variation by gene duplication and inactivation seems to be important for evolution of chemosensation (Nei et al. 2008). The situation with UGT in plants might be similar. Assuming that the endogenous secondary metabolism in plants is the main force driving the evolution of UGT, large species differences can be expected. Gene families that are rapidly evolving such as the terpene synthases (Chen et al. 2011) and, especially, the cytochrome P450 (Nelson and Werck-Reichhart 2011) will create a challenge for plants to also evolve UGT conjugating new metabolites to prevent self toxicity. In the case of rice and *B. distachyon*, the CYP gene numbers are 329 and 226, respectively. Some families are expanding more rapidly than others: in the CYP71 gene family, 190 genes are present in rice and 113 in *B. distachyon*, with only less than 20% seemingly orthologous genes. The differences in the metabolome that are expected to be caused by this difference in cytochrome P450 repertoire should also have an impact on the evolution of the UGT gene family, most likely to a much higher extent than xenobiotics such as the *F. graminearum* toxins.

In conclusion, this study has identified a UGT gene family in monocotyledonous species, which contains genes that encode the ability to metabolize the *Fusarium* toxin DON. Although all of these genes are induced by the toxin, only two of the six genes in this gene family conjugate DON to D3G. Potentially, other DON-responsive genes could accept related xenobiotics as substrates, which induce a similar response in gene expression. We show that not necessarily the closest homologs share the same substrate specificity. However, the proposed function of UGT can be easily tested by heterologous expression in yeast.

MATERIALS AND METHODS

Cloning and heterologous expression of UGT genes in yeast.

Coding sequences of *B. distachyon* UGT genes Bradi5g-02780.1, Bradi5g03300.1, Bradi5g03370.1, Bradi5g03380.1, Bradi5g03390.1, and Bradi5g03400.1 predicted by the MIPS Brachypodium database; the *O. sativa* UGT NM_001058778.1, NM_001058779.1, and NM_001058780.1; and the *S. bicolor* UGT XM_002447461.1 were optimized for expression in *Saccharomyces cerevisiae* by reverse translating the amino acid sequence utilizing the codon usage table for *S. cerevisiae* (according to codon tables provided by the Kazusa DNA Research Institute). Codon optimized genes were custom synthesized (*B. distachyon* UGT: MrGene, Regensburg, Germany, now Invitrogen, Carlsbad, CA, U.S.A.; and rice or sorghum UGT: Eurofins MWG Operon, Ebersberg, Germany), including flanking 5' HindIII and 3' NotI restriction sites (Supplementary Table S5). ORF were cloned into the HindIII/NotI cloning sites of yeast expression vector pYAK7 in frame behind the N-terminal c-Myc epitope tag (2 μ -P_{ADHI}-C-Myc-MCS-T_{ADHI}, LEU2) (Poppenberger et al. 2003). Yeast strain YZGA515 was transformed with each of the constructs and additionally with the previously reported DON-UGT genes *DOGT1* (pBP868) (Poppenberger et al. 2003) and *HvUGT13248* (pWS1921) (Schweiger et al. 2010) in the same vector backgrounds as positive controls. As a negative control, we transformed YZGA515 with the empty pYAK7 plasmid pBP910. Transformants were selected on synthetic complete medium lacking leucine. Bd2g04720.1 and Bd2g04760.1 were PCR amplified from *B. distachyon* cDNA using primers that

extended the PCR product at both ends with approximately 50 bp of sequence identical to the region flanking the multiple cloning site of pYAK7. The plasmids were generated by homologous recombination in vivo. Strain YZGA515 was transformed with a NotI-linearized pYAK7-derived vector (200 ng, pGW1013), which harbors the yeast *sup4-o* gene in the multiple cloning site together with each fragment in equimolar amounts or higher ratios (1:1, 1:2, 1:10, and 1:40). The recombinant plasmids were recovered from yeast and the correct recombination event was confirmed by restriction digest of the isolated plasmids. The presence of the transfer (t)RNA suppressor allowed phenotypic selection of strains harboring successful recombination events directly on the transformation plate. If the amber suppressor tRNA was not removed by the recombination event, the *ade2-101* allele present in the yeast host was suppressed by *sup4-o* and white colonies were formed. The *sup4-o* in the high-copy plasmid is toxic for yeast, leading to a small number of transformants yielding small white colonies, whereas large red colonies were formed if the desired recombination event replaced the *sup4-o* insert. A list of the constructs and the corresponding primers is given in Supplementary Table S6. Immunodetection was conducted using a primary mouse anti-c-Myc antibody (1:5000, clone 9E10; Invitrogen) and a horseradish peroxidase-conjugated secondary anti-mouse antibody (Jackson, West Grove, PA, U.S.A.). The SuperSignal West Pico chemiluminescent substrate was used for signal detection (Pierce, Rockford, IL, U.S.A.).

Phenotypic testing and feeding experiments.

Crystallized DON was dissolved in water to a stock concentration of 10 g/liter. YPD plates containing different amounts of DON were used to spot dilutions of individual transformants expressing either UGT or vector controls. For this purpose, freshly transformed strains were grown in synthetic complete media lacking leucine to exponential phase (optical density at 600 nm [OD₆₀₀] of 0.7) and diluted to OD₆₀₀ of 0.05 and 0.01 with fresh selective medium. Cell suspensions (3 μ l) were spotted onto plates and incubated at 30°C for 3 days.

Strains conferring resistance to DON in the plate assay were again cultivated in larger volumes of liquid selective media in five biological replicates. Cells were harvested in exponential phase and washed repeatedly in fresh medium. For each sample 5 ml of cell suspension with an OD₆₀₀ of 4 was incubated at 30°C with DON at 200 mg/liter. DON concentration was raised to 350 mg/liter after 1 h and to a final concentration of 500 mg/liter 2 h after the start of the experiment. In total, 400 μ l was collected by centrifugation 8, 16, and 24 h after the first DON addition. Cells were washed twice with ice-cold distilled water and extracted by adding 1.25 ml of a 4°C methanol/H₂O (4:1, vol/vol) mix. Samples were frozen at -80°C for 15 min and sonicated on ice for 15 min. After centrifugation, both the supernatant and 500 μ l of the extract were stored at -20°C. For HPLC/MS-MS analysis, thawed samples were diluted with 10% aqueous methanol. The intra- and extracellular contents of DON and D3G were measured as described by Berthiller and associates (2009) using electrospray negative ionization on a 4000 QTrap HPLC/MS-MS system (Applied Biosystems, Foster City, CA, U.S.A.).

Plant growth conditions.

B. distachyon ecotype Bd21 was cultivated in a growth chamber under a 20-h light period at 23 $\pm$ 2°C under fluorescent light (265 μ E m⁻² s⁻¹ at the soil level and approximately 315 μ E m⁻² s⁻¹ at the spike level). Prior to sowing, seed were surface sterilized by incubation in a 0.6% sodium hypochlorite solution for 10 min with gentle shaking, followed by three rinses in sterile distilled water. Sterilized seed were subse-

quently incubated for 5 days at 4°C in the dark. Plants were grown routinely on a 3:1 mixture of compost (Tref terreau P1; Jiffy France SARL, Trevoux, France) and standard perlite (Sinclair, Gainsborough, UK), soaked with an aqueous solution containing a carbamate fungicide (Previcur at 2 ml/liter; Bayer Crop Sciences, Lyon, France) and a larvicide (Hortigard at 1 g/liter; Syngenta France, Guyancourt, France). Plants were usually watered at 2- to 3-day intervals using a standard nutritional solution and were never allowed to stand in water.

***F. graminearum* strain maintenance and spore production.**

F. graminearum strains PH-1 and *Δtri5* (MU102 mutant strain) (Cuzick et al. 2008) were maintained on potato dextrose agar (PDA) plates. To obtain spores, 2- to 4-mm² plugs from 15-day-old PDA plates were inoculated in liquid mung bean medium (10 plugs for 20 ml) (Bai and Shaner 1996) and incubated at 150 rpm at room temperature for 5 to 6 days. The resulting spore suspension was then diluted 10 times in fresh liquid mung bean medium and further incubated for five to six additional days under the same conditions. For pathogenicity assays, spores were further filtrated onto sterile Miracloth (Calbiochem, Toulouse, France) and resuspended in 0.01% Tween 20 at a final concentration of 10⁵ spores/ml.

Pathogenicity assays and DON application.

Inoculation was performed by pipetting 300 spores (3 μl of a 10⁵ spores/ml suspension) into a central floral cavity of the second spikelet starting from the top of the spike of Bd21 plants at mid-anthesis (approximately 30 to 35 days after sowing). A single spike was inoculated per plant to further guarantee the independence of the samples. Inoculated plants were covered with clear plastic bags sprayed with distilled water beforehand. For the first 24 h, inoculated heads were kept in the dark, then incubated with a photoperiod of 16 h of light and 8 h of darkness at 20°C with light intensities similar to those used for plant development (discussed above). Similarly, DON was applied as an acetonitrile solution (2 μg/cavity) and plants were incubated as after fungal inoculation, except that they were placed directly in light or dark conditions. Applications of 0.01% Tween 20 or acetonitrile were performed as control conditions for fungal infection or DON treatment, respectively.

RNA extraction.

Three experimental repetitions were performed, differentiated by different sowing dates. In each experimental repetition, up to six inoculated spikelets per time point were collected at 0, 3, 8, 12, 24, and 48 h after DON application or at 0, 24, 48, 72, 96, and 168 h after fungal inoculation (PH-1 or *Δtri5* strain). Spikelets originated from independent plants and were pooled for RNA extraction. For each sample, spikelets were ground in liquid nitrogen and total RNA was extracted from 0.1 g of the resulting powder using TRIzol (Invitrogen, Life Technologies SAS, Saint-Aubin, France) followed by an RNase-free DNase I step (Ambion; Applied Biosystems, Courtaboeuf, France) according to the manufacturers' instructions. Total RNA was further purified using the NucleoSpin RNA Clean-up XS kit (Macherey-Nagel, Hoerd, France).

Real-time PCR.

cDNA synthesis was performed on 1 μg of total RNA using the ImProm-II reverse-transcription system (Promega France, Melun-les-Charbonnières, France) according to the manufacturer's instructions. The resulting product was diluted 10 times in nuclease-free water. Primers were designed to amplify 10 gene transcripts, including two reference genes Bradi4g00660 (*UBC18*) and Bradi4g41850 (*ACT7*, now referred to as *ACT3*-like under accession number XM_003578821 in the nucleotide

National Center for Biotechnology Information database) as previously determined by Hong and associates (2008) (Supplementary Table S7). qPCR reactions were performed on 2 μl of the diluted cDNA product using 800 pmoles of each specific primer and 10 μl of SYBRGreen Master Mix in a final volume of 20 μl. Reactions were performed in a Light Cycler LC480 Real-time PCR system (Roche Diagnostics, Meylan, France). All qRT-PCR reactions were carried out on biological triplicates, each in technical duplicate. The final threshold cycle (Ct) values were the mean of six values (biological triplicates, each with technical duplicate). The comparative $\Delta\Delta C_t$ method was used to evaluate the relative quantities of each amplified product in the samples. The Ct was automatically determined for each reaction by the Light Cycler LC480 Real-time PCR system set with default parameters. The specificity of the qRT-PCR reactions was determined by melt curve analysis of the amplified products using the standard method installed in the system.

dN/dS-analysis.

To identify positively selected sites, homologous *B. distachyon* UGT were grouped based on the clusters identified via the phylogenetic comparison with *A. thaliana*. Gap-free codon alignments were generated using PAL2NAL (Suyama et al. 2006). In a prior analysis, we screened for possible recombination breakpoints that were taken into account in subsequent analyses (GARD algorithm) Kosakovsky Pond et al. 2006). Alignments of full-length genes and N- and C-terminal regions (each taking into account the terminal 250 amino acids) were first analyzed using PARRIS (Scheffler et al. 2006). Single sites under positive selection were identified using the SLAC/REL and FEL-algorithms (Kosakovsky Pond et al. 2005), which are all part of the computational phylogenetics software package HyPhy provided by the School of Medicine of the University of California, San Diego (Delpert et al. 2010; Pond et al. 2005). Codon positions with a dN/dS-ratio of 1 or higher identified with at least one of the applied algorithms were considered under positive selection.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

The *Arabidopsis* Cytochrome P450, Cytochrome_{b5}, P450 Reductase, β -Glucosidase, and Glycosyltransferase database: www.p450.kvl.dk
 Kazusa DNA Research Institute Codon Usage database: www.kazusa.or.jp/codon
 The Munich Information Center for Protein Sequences (MIPS): www.helmholtz-muenchen.de/en/ibis
 OrthoMCL database: www.orthomcl.org
 School Of Medicine of the University of California, San Diego
 Datamoney server: www.datamoney.org