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The *Brachypodium distachyon* UGT Bradi5gUGT03300 confers type II fusarium head blight resistance in wheat

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Fusarium head blight (FHB), caused by fungi belonging to the *Fusarium* genus, is a widespread disease of wheat (*Triticum aestivum*) and other small-grain cereal crops. The main causal agent of FHB, *Fusarium graminearum*, produces mycotoxins mainly belonging to type B trichothecenes, such as deoxynivalenol (DON), that can negatively affect humans, animals and plants. DON detoxification, mainly through glucosylation into DON-3-O-glucose, has been correlated with resistance to FHB. A UDP-glucosyltransferase from the model cereal species *Brachypodium distachyon* has been shown to confer resistance both to initial infection and to spike colonization (type I and type II resistances, respectively). Here, the functional characterization of transgenic wheat lines expressing the *Bradi5g03300 UGT* gene are described. The results show that, following inoculation with the fungal pathogen, these lines exhibit a high level of type II resistance and a strong reduction of mycotoxin content. In contrast, type I resistance was only weakly observed, although previously seen in *B. distachyon*, suggesting the involvement of additional host-specific characteristics in type I resistance. This study contributes to the understanding of the functional relationship between DON glucosylation and FHB resistance in wheat.

Keywords: Brachypodium, deoxynivalenol, detoxification, fusarium head blight, wheat

Introduction

In the last decade, Europe has produced an average of 227 million tonnes of wheat each year (FAOSTAT, http://www.fao.org/faostat/en/#data/QC), positioning this continent as one of the largest world wheat growers. Agricultural biotechnology has provided a lot of solutions for the gain of resistance to biotic stresses and tolerance to abiotic stresses. However, several biotic stresses, such as wheat scab, still result in a deterioration of yield and grain quality. Frequent in European bread wheat varieties, wheat scab or fusarium head blight (FHB), which is caused by fungi of the genera *Fusarium* and *Microdochium*, is one of the most important diseases of small-grain cereal crops (Nicholson *et al.*, 2003). The main FHB causal agent, *Fusarium graminearum*, can produce type B trichothecene (TCT B) mycotoxins, such as deoxynivalenol (DON). These molecules negatively affect humans, plants and animals. Indeed, TCT B can interact with the 60S ribosomal subunit (Fried & Warner, 1981) and subsequently inhibit eukaryotic protein synthesis. Further effects are the modification of DNA and RNA biosynthesis, the stimulation of programmed cell death under high DON concentrations (Desmond *et al.*, 2008) and the regulation of MAP kinase pathways (Rocha *et al.*, 2005). Decreasing trichothecene concentration in grains is necessary for food safety. The European Union has authorized a maximum tolerance level of 1250 ppb for DON in non-processed products intended for the adult human diet. Tolerated levels of DON are even lower for processed products (200–750 ppb, Commission Regulation (EC) no. 1881/2006).

To date, sources of resistance conferring complete resistance to FHB have not been identified. There is no monogenic resistance to FHB, resistance described so far being only quantitative. Numerous quantitative trait loci (QTLs) have been identified in *Triticum aestivum* using different wheat varieties, located all over the genome (Steiner *et al.*, 2017). The major ones are on chromosome 3BS (*Fhb1*), 5AS (*Qfhs.ifa-5A*) and 6BS (*Fhb2*) (Buerstmayr *et al.*, 2014). Further QTLs include *Fhb4* and *Fhb5*, *Qfhs.nau-2DL* and *Fhb7* in *Thinopyrum ponticum* and numerous other minor QTLs throughout the genome (Buerstmayr *et al.*, 2009). Two main types of FHB resistance have been described: type I refers to the resistance to the initial infection, type II corresponds to the resistance to the spread of the fungal pathogen along the spike (Boutigny *et al.*, 2008). Among all FHB resistance QTLs identified in wheat, the *Fhb1* locus seems to

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explain 60% of the phenotype variation for type II FHB resistance (Buerstmayr *et al.*, 2009; Steiner *et al.*, 2017) and also for toxin accumulation.

Little information is currently available concerning genes involved in resistance. Based on the cloning of *Fhb1*, a gene encoding a pore-forming toxin-like (PFT) protein located in the *Fhb1* region was reported to be responsible for the *Fhb1*-associated FHB resistance in wheat (Rawat *et al.*, 2016). However, these findings could not be confirmed in a more recent study (Jia *et al.*, 2018) and the exact nature of the *Fhb1* QTL is still under debate. Comparative transcript analyses identified up-regulated genes encoding proteins fulfilling various functions such as 4-coumarate CoA ligase (4CL), callose synthase (CS), basic helix loop helix (Bhlh041) transcription factor, glutathione *S*-transferase (GST), ABC transporter-4 (ABC4) and cinnamyl alcohol dehydrogenase (CAD) in recombinant inbred lines carrying resistant alleles of QTL-*Fhb2* (Dhokane *et al.*, 2016). The overexpression of *TaFROG*, an orphan gene, enhanced DON and FHB resistance in wheat (Perochon *et al.*, 2015). TaBCC3.1, an ABC transporter, was shown to contribute to DON tolerance (Walter *et al.*, 2015) as well as a methionyl-tRNA synthetase gene (*TaMETRS*) (Zuo *et al.*, 2016). Moreover, other studies correlated morphological and phenological traits with FHB resistance. The semi-dwarfing alleles *Rht-D1b* and *Rht-B1b* (Voss *et al.*, 2008; Lu *et al.*, 2013) have been correlated with increased anther retention and FHB severity.

Several studies have associated the ability to conjugate DON into DON-3-*O*-glucose (D3G) with partial resistance towards FHB (for a review, see Gunupuru *et al.*, 2017). In particular, Lemmens *et al.* (2005) showed that the presence of the *Fhb1* locus was correlated with increased metabolizing of DON into D3G.

This research focuses on glycosylation, which is catalysed by a family of enzymes named UDP-glycosyltransferases (UGTs). These enzymes are able to add a molecule of sugar, previously activated by a uridine diphosphate (UDP; UDP-glucose, UDP-rhamnose, UDP-galactose, UDP-xylose, UDP-glucuronic acid (mainly in mammals)), onto liphophilic molecules and are involved in different functions including hormone homeostasis, detoxification of xenobiotics, biosynthesis and storage of secondary metabolite compounds (Gachon *et al.*, 2005). In *Arabidopsis*, UGT73C5 has been shown to be involved in the glucosylation of DON and the overexpression of the corresponding gene in transgenic plants was able to confer resistance to DON (Poppenberger *et al.*, 2003). Recent research (Schweiger *et al.*, 2010) showed how upon heterologous expression of full-length cDNAs, only one gene, the *HvUGT13248* gene encoding a candidate barley glucosyltransferase, conferred DON resistance in yeast. Moreover, transgenic wheat expressing the barley UDP-glucosyltransferase (HvUGT13248) showed FHB variable resistance and a diminution in the disease phenotypes under field conditions (Li *et al.*, 2015). A wheat UGT gene *TaUGT12887*, associated with the locus *Qfhs.ifa-5A* for FHB resistance, was also shown to provide DON tolerance when expressed in a yeast strain (Schweiger *et al.*, 2013b). More recently, the *Brachypodium distachyon* Bradi5g03300 UGT was shown to confer tolerance to DON through glucosylation of DON into D3G *in planta* and to be involved in quantitative resistance to FHB (Pasquet *et al.*, 2016). Functional analysis of this gene showed increased root sensitivity of mutant lines to the toxin. Conversely, the overexpression lines showed a higher root tolerance to the toxin and an increased quantitative resistance to FHB.

To further compare wheat and *Brachypodium* interaction with *F. graminearum* and characterize more precisely the involvement of DON glucosylation in FHB resistance in wheat, the goals of this study were to introduce the *B. distachyon Bradi5g03300* gene into the spring wheat variety Apogee susceptible to FHB, and conduct phenotypic analyses on transgenic lines to determine whether expression of this gene increases resistance to FHB and root tolerance to DON in wheat.

Materials and methods

Plant material and growth conditions

Apogee and transgenic lines were cultivated in a growth chamber under a 16 h light period at 24 ± 5 °C under fluorescent light (265 μmol m^{-2} s^{-1} at the soil level). Seeds were surface sterilized by incubation in 0.6% sodium hypochlorite solution for 3 min with gentle shaking followed by three rinses for 10 min in sterile distilled water, and incubated for 4 days at 4 °C in the dark. Plants were grown on a 2:1 mixture of compost (Tref terreau P1, Jiffy France SARL) and standard perlite (Sinclair), soaked with an aqueous solution containing a carbamate fungicide (Previcur at 2 mL L^{-1}; Bayer Crop Sciences) and a larvicide (Hortigard at 1 g L^{-1}; Syngenta France). Plants were watered at 3-day intervals using a standard nutrient solution.

Culture medium preparation

For the culture medium, 4 g plant agar Kalys HP 696-1, 1.1 g basal salt mixture and 5% sucrose were mixed with water, filled up to 500 mL and pH adjusted to 5.7. The medium was then autoclaved for 20 min at 120 °C. To study root tolerance to the mycotoxin, DON was added to the medium at a final concentration of 10 μM (2.96 ppm). To test for segregation of the transgene, the medium contained 2% mannose as sole carbon source.

In vitro seed germination and root development of *T. aestivum*

Surface-sterilized seeds were placed onto Whatman paper wetted with sterile distilled water in closed Petri dishes and incubated for 24 h in the dark at 10 °C. Selected homogeneously germinated wheat grains were inoculated onto 400 μL of 50% Murashige & Skoog (MS) with the embryo of the grain facing up in a 2 mL tube. Tubes were then moved into a sterile box to preserve axenic conditions and were incubated for 48 h in the dark at 24 °C, after which root measurements were started. The box was then moved into a 16 h light period at

24 ± 5 °C under fluorescent light (265 μmol m^{-2} s^{-1} at the soil level). Day 0 starts from the set-up of small seedlings into Eppendorf tubes.

Construction and Apogee transformation

The *B. distachyon Bradi5g03300* gene was introduced through biolistics (Tassy *et al.*, 2014) into the wheat variety Apogee, susceptible to FHB. The construction was obtained by a multisite Gateway system (Life Technology). Three entry vectors were used: the first containing the *Zea mays* ubiquitin promoter region, the second containing *Bradi5g03300* cDNA and the third the *NOS* terminator sequence and the *PMI* gene for selection, which encodes phosphomannose-isomerase that converts mannose-6-phosphate to fructose-6-phosphate under control of the *Z. mays* ubiquitin promoter region and the *NOS* terminator sequences. These three vectors were recombined in the destination vector pDEST R4-R3 with clonase to obtain the expression clone, and the plasmid was purified. The *Bradi5g03300* gene and PMI selection cassette were isolated by digestion with *Not*I restriction endonuclease as described by Tassy *et al.* (2014). One thousand immature embryos from the cultivar Apogee were then bombarded using gold beads coated with a *Not*I-linearized double-strand DNA fragment generated from the plasmid construct (allowing expression of the *Bradi5g03300* gene as well as expression of the *PMI* gene; Fig. S1). In the selection system using the *PMI* gene, only transformed cells are able to use mannose as sole carbon source. After selection of transformants in MS medium containing 2% mannose, segregation analyses were used to identify homozygous lines.

Fusarium graminearum strains, maintenance and spore production

Fusarium graminearum strains PH-1, further named FgDON$^+$ (DON/15ADON chemotype) and FgDON$^+$-GFP (a DON$^+$ transformant constitutively expressing GFP under the control of the *Pyrenophora tritici-repentis ToxA* promoter region (Pasquet *et al.*, 2016)) were maintained on potato dextrose agar plates. To obtain fungal spores, 2–4 mm^2 plugs from 15-day-old potato dextrose agar plates were inoculated in liquid mung bean medium (10 plugs for 20 mL) and incubated under agitation (150 rpm) at room temperature for 5–6 days under the same conditions. The final volume of mung bean broth was inoculated with 1/10 volume of the first suspension of spores and incubated again for 5–7 days under agitation (150 rpm) at room temperature. For pathogenicity assays, spores were filtrated onto sterile Miracloth (Calbiochem) and resuspended in 0.01% Tween 20 at a final concentration of 10^5 spores mL^{-1}.

Pathogenicity assays

Inoculation was performed by pipetting 500 spores (5 μL of a 10^5 spores mL^{-1} suspension) of either of the two strains into a central floral cavity of the secondary spikes at mid-anthesis (BBCH65). Alternatively, whole spikes were sprayed with the fungal spore suspension (10^5 spores mL^{-1}) until dripping. Two secondary spikes were inoculated per plant for both inoculation methods. For the first 24 h, inoculated heads were kept in the dark, then incubated with a photoperiod of 8 h light/16 h dark at 20 ± 2 °C with the same light intensity used for the plant development. Application of 0.01% Tween 20 was used as a control.

Epifluorescence microscopy

Following point inoculation with the FgDON$^+$-GFP strain as described above, infected spikes were cut longitudinally and pictures were taken with the stereomicroscope MZ16F LEICA using the GFP-specific large filter, allowing the observation of chlorophyll autofluorescence in red.

DNA extraction

For quantification of *F. graminearum* DNA, five spikes spray-inoculated with FgDON$^+$ strain were pooled per time point (14 days after inoculation). Whole genomic DNA was extracted as described by Pasquet *et al.* (2016).

RNA extraction and cDNA synthesis

Leaves, roots and spikes from 4-week-old independent plants or infected material were ground in liquid nitrogen, and total RNA was extracted from 0.1 g of the resulting powder using TRIzol (Invitrogen) followed by an RNase-free DNase I step (TURBO DNase; Ambion) according to the manufacturer's instructions. cDNA synthesis was performed on 1 μg total RNA using the ImProm-II reverse transcription system (Promega). The product was diluted 10-fold in nuclease-free water before use.

Reverse transcription-quantitative PCR (RT-qPCR) and fungal DNA quantification by qPCR

The *Bradi5g03300* cDNA was amplified using specific primers (Table S1). The wheat actin gene (*TRIAE_CS42_1AS_TGACv1_020044_AA0074210*) was used as a reference gene in these experiments (Table S1). RT-qPCR was performed on 2 μL of the diluted cDNA product using 8 pmol of each specific primer and 10 μL of SYBRGreen Master Mix in a final volume of 20 μL. Reactions were performed in a LightCycler LC480 real-time PCR system (Roche). All RT-qPCR were carried out on biological triplicates, each in technical duplicate. The comparative C_t method was used to evaluate the relative quantities of each amplified product in the samples. The specificity of the RT-qPCR amplification was determined by melt-curve analysis of the amplified products using the standard method installed in the system. In addition, Apogee was kept as a control line. Transgene expression was calculated as follows: C_t(transgene)/C_t(control gene: ACT) × 100.

Quantification of fungal DNA was realized by quantitative PCR (qPCR) on 20 ng total DNA using primers specific for the fungal 18S ribosomal subunit-encoding genomic region (Pasquet *et al.*, 2016). Reactions were performed in a LightCycler LC480 real-time PCR system. The absolute quantity of fungal DNA in each sample was determined by comparing with a standard range of fungal genomic DNA alone.

Mycotoxin extraction and analysis

Fresh ground material (500 mg of spikes or spikelets infected by the FgDON$^+$ strain) was extracted with 7 mL of acetonitrile/water (84:16, v/v) for 1 h at room temperature on a tube rotator (50 rpm). Before extraction, 0.5 μg of fusarenon X was added to each sample as an internal standard. After centrifugation (5 min at 5000 ***g***), the supernatant was purified on Trichothecene P Columns (P51 R-Biopharm) and 3 mL of filtrate were evaporated at 50 °C in a dryness of nitrogen. The pellet

was resuspended in 400 μL of methanol/water (50:50, v/v) and filtered through a 0.20 μm filter before analysis. DON, D3G, 15-ADON and fusarenon X concentrations were determined using HPLC-MS/MS analyses. These analyses were performed using a micro-TOF II (Brukner) equipped with an Acquity UPLC (Waters), a reverse phase Kinetex 1.7 μm XB-C18 column (100 × 2.1 mm; Phenomenex) maintained at 45 °C and an ESI source. Solvent A consisted of methanol/water (10:90, v/v) and solvent B consisted of methanol/water (90:10, v/v). The flow rate was kept at 400 μL min^{-1} and went to the electrospray source. Gradient elution was performed with the following conditions: 2 min with a linear gradient from 80% to 5% A, 2 min held at 5% A, 1 min linear gradient from 5% to 80% A and 80% A for 2 min post-run reconditioning. The injection volume was 5 μL. The electrospray interface was used in the negative ion mode at 195 °C with the following settings: nebulizer gas, 4 bar; dry gas, 8.5 L min^{-1}; ion spray voltage, −3700 V. Quantification was performed using external calibration with DON (Sigma-Aldrich), D3G (Sigma-Aldrich), 15-ADON (Sigma-Aldrich) and fusarenon X standard solutions, ranging from 10 to 1000 ng mL^{-1}.

Results

Generation of lines expressing the *Bradi5g03300* gene

A *Not*I fragment carrying the *B. distachyon Bradi5g03300* cDNA under the control of the *Z. mays* ubiquitin promoter, the same expression cassette as the one previously used in *B. distachyon* (Pasquet *et al.*, 2016), as well as a phosphomannose isomerase (PMI) expression cassette, was introduced through biolistics (Tassy *et al.*, 2014) into the wheat variety Apogee, susceptible to FHB (Fig. S1). Transgenic wheat plants were regenerated from selected transformed immature embryo-derived calli on media containing various concentrations of mannose, based on the PMI selection, according to the protocol described by Wright *et al.* (2001). After selection, seven putative primary transformants (T_0) were recovered and grown up to maturity to collect seeds. These plants were tested for expression of the *Bradi5g03300* gene. Only line 3 did not express the transgene, although it expressed the *PMI* selection marker (Fig. S2a,b). Due to technical problems, only three out of these six primary transformants could be recovered and grown to maturity. These plants (T_1) were tested for the presence and expression of the *Bradi5g03300* gene. Segregation tests were performed for each transformant with a screening *in vitro* on mature seeds (minimum 60–80 seeds) of T_2 transformed lines, based on the ability to grow on mannose as sole carbon source. Homozygous transformed line 2 was obtained at the T_2 step while homozygous lines 4 and 7 were recovered one generation later. Line 3, which would have been the most rigorous control line, could not be used because of poor seed production (data not shown). The wildtype nontransformed Bd21-3 ecotype was therefore used as a control in all experiments.

To determine if the overexpression of the *Bradi5g03300* gene in *T. aestivum* alters development and overall morphology compared to the control, plant height, the number of tillers at 6, 14, 27 and 39 days after sowing, the number of spikelets per spike 56 days after sowing (the mid-anthesis or BBCH65 stage in these conditions) and thousand-grain weight (TGW) on plants grown in the greenhouse were measured. No major developmental modification was shown in the transgenic lines (Fig. S3). Nevertheless, a slight decrease in TGW was observed in lines 4 and 7 compared to the control (Fig. S3d, one-way analysis of variance and Duncan multiple range test ($\alpha = 0.05$), $n = 1000$), positively correlated with the transgene expression in all three organs, in particular in spikes (Fig. 1f for spikes, Fig. 4c for roots, Fig. S4 for leaves).

Expression of *Bradi5g03300* in *T. aestivum* and type II resistance to FHB

Bradi5g03300 relative expression was analysed in the three independent homozygous lines, each carrying the construct mentioned earlier. RT-qPCR experiments were conducted to determine whether lines expressed the *Bradi5g03300* gene in spikes (Fig. 1f). Results showed that the three transformants exhibited different levels of transgene expression in spikes. Line 4 showed the highest *Bradi5g03300* relative expression in spikes followed by line 7 and line 2.

The transformed lines were then analysed at the phenotypic level to determine whether expression of the *B. distachyon Bradi5g03300* gene confers FHB resistance. Plants were point inoculated at mid-anthesis using an *F. graminearum* strain able to produce DON ($FgDON^+$). The Apogee variety was used as a control. The percentage of spikelets with symptoms was scored 7 and 14 days post-inoculation (dpi). Percentage of spikelets with symptoms at 7 dpi was 36% for the control, 9% for line 4, 16% for line 7 and 27% for line 2 (pairwise *t*-tests, $P < 0.05$, $n > 30$; Fig. 1a). Percentage of spikelets with symptoms at 14 dpi was 79% for the control, 27% for line 4, 34% for line 7 and 71% for line 2 (pairwise *t*-tests, $P < 0.05$, $n > 30$; Fig. 1c). Typical symptoms are shown in Figure 1b,d. These results showed that transgenic wheat lines expressing the *Bradi5g03300* gene exhibited a significantly higher resistance to FHB compared to the nontransformed control Apogee plants. The most resistant transformed line was line 4, exhibiting the highest expression level of the *B. distachyon Bradi5g03300* transgene (Fig. 1f). These results were confirmed by quantification of fungal genomic DNA in infected spikes by qPCR. Fungal biomass at 14 days was shown to be 21 times lower (95.1% reduction) in line 4 in comparison to Apogee. Infected spikes of lines 7 and 2 contained 13 and 2.5 times less fungal DNA, respectively (92.1% and 61.4% reduction, respectively), in comparison to Apogee (Fig. 1e).

To investigate how expression of the *Bradi5g03300* transgene altered fungal development, spikes of transformant line 4 exhibiting the highest expression level and Apogee as a control were point inoculated with a transformant of the $FgDON^+$ strain constitutively expressing

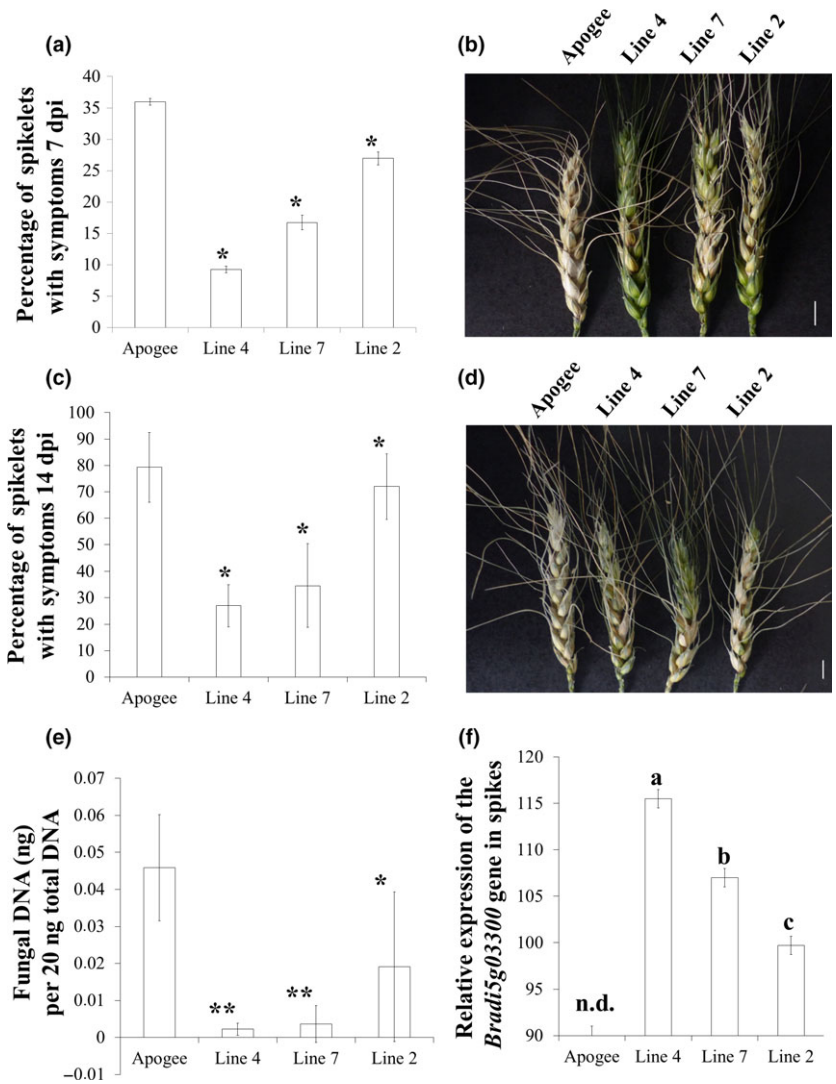

Figure 1 Expression of the *Bradi5g03300* gene increases resistance to the spread of fusarium head blight (FHB). Percentages of spikelets exhibiting FHB symptoms on the entire inoculated spikes, 7 days (a) and 14 days (c) after point inoculation by fungal strain FgDON$^+$. The asterisks indicate significant differences between Apogee (control line) and each transgenic line; data represent mean values of three independent biological experiments with a minimum of 30 spikes per genotype and per replicate, error bars represent the standard deviation; pairwise *t*-tests, $P < 0.05$ ($n > 30$). Photographs show typical symptoms observed on transgenic lines and on control (Apogee) 7 days (b) and 14 days (d) after point inoculation by the FgDON$^+$ strain. Bar = 1 cm. (e) Quantification of fungal DNA by qPCR 14 days after point inoculation of spikes by the FgDON$^+$ strain. Data represent mean values of three independent biological experiments, error bars represent the standard deviation. Asterisks indicate significant differences between Apogee (control line) and each transgenic line as determined by pairwise *t*-tests, $P < 0.005$ (**) and $P < 0.05$ (*). (f) Relative expression of the *Bradi5g03300* gene in spikes of wheat transgenic lines. The relative quantity of gene transcripts was calculated using C_t(transgene)/C_t(control gene) × 100. The wheat *ACT* gene was used as endogenous control to normalize the data for differences in input RNA between different samples. Data represent mean values of three independent biological experiments, error bars represent the standard deviation. Different letters indicate significant differences; one-way analysis of variance and Duncan multiple range test ($\alpha = 0.05$). n.d., not determined (nontransformed control). [Colour figure can be viewed at wileyonlinelibrary.com].

GFP (FgDON$^+$-GFP). Despite the disturbance caused by autofluorescence in the anthers, visualized by a yellow-green fluorescence, a delay in colonization of adjacent spikelets was clearly observed in line 4 compared to the control (Fig. 2). No obvious difference could be detected at the early 4 dpi time point, where only the inoculated floret was fluorescent in both plant genotypes (Fig. 2, left panels). However, differences between the two genotypes were observable at 7 dpi. Indeed, in the susceptible line Apogee, the fungus had colonized several adjacent florets following development in the rachis, which appeared green fluorescent (Fig. 2, right bottom panel). In contrast, at this latter time point in the transformed line 4, fluorescence was mostly restricted to the inoculated floret (Fig. 2, right top panel), which is typical of type II FHB resistance.

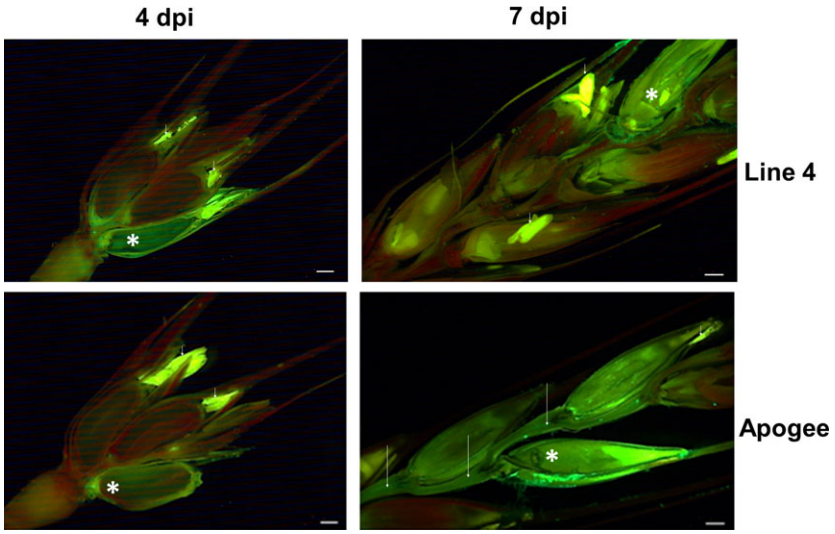

Figure 2 Expression of the *Bradi5g03300* gene reduces spikelet colonization by the FgDON$^+$ strain. Micrographs showing longitudinally sectioned spikes of Apogee and line 4, 4 days (left) and 7 days (right) after point inoculation with the GFP-tagged *Fusarium graminearum* FgDON$^+$ strain, visualized with epifluorescence illumination. Inoculated floral cavities are indicated by white asterisks. Anthers autofluorescence is shown by white arrowheads. Green fluorescence due to fungal development in the rachis of the Apogee spike (right bottom panel) is indicated by white arrows. Bars = 5 mm.

Expression of *Bradi5g03300* in *T. aestivum* and type I resistance to FHB

The previous results showed that transgenic wheat expressing the *B. distachyon UGT* gene exhibited significantly higher resistance to fungal spike colonization compared to the control line and that this phenotype positively correlated with level of relative expression of the transgene. Spray inoculations were performed on the same plant lines to determine whether expression of *Bradi5g03300* also conferred resistance to initial infection, as already observed in *B. distachyon* (Pasquet *et al.*, 2016). Differences between the transformed lines and the Apogee control could be observed, but were far less pronounced. Percentage of spikelets with symptoms at 7 dpi was 15.8% for the control, 11.3% for line 4, 18.2% for line 7 and 17.9% for line 2 (pairwise *t*-tests, $P < 0.05$, $n > 30$; Fig. 3), and differences to the control were only statistically significant for line 4. At 14 dpi, all three transgenic lines exhibited a significantly lower percentage of symptoms than the control line, with 17.1%, 21.2% and 27.9% spikelets with symptoms, respectively, compared to 35.2% for the control line Apogee (pairwise *t*-tests, $P < 0.05$, $n > 30$; Fig. 3).

Expression of *Bradi5g03300* in *T. aestivum* and root tolerance to DON

DON has already been reported to affect root growth in wheat. To determine whether *Bradi5g03300* expression modifies root tolerance to DON, root growth of the different transformed lines and of the Apogee variety was determined on 50% MS either with or without the addition of 10 μM DON. While no significant difference in root development was observed between the transformed lines and Apogee (control line) on agar medium without DON (Fig. 4a, left), strong phenotypes were observed on 10 μM DON after 7 days growth (Fig. 4a, right). Growth inhibition of the primary seminal root was around 11% for line 4, 49% for line 7 and 55% for line 2, while it was around 70% for Apogee (Fig. 4b, pairwise *t*-tests, $P < 0.05$, $n > 30$). As previously observed in FHB resistance assays, the impact of DON on root growth is inversely correlated with the transgene expression level in this organ (Fig. 4c). Indeed, line 4 shows a nearly full resistance to DON at this concentration, whereas the other two transformed lines exhibit intermediate root growth inhibition between line 4 and the control line Apogee.

Bradi5g03300 expression in *T. aestivum* and decreased levels of total DON in infected spikes

To determine if the enhanced type II resistance conferred by *Bradi5g03300* was correlated with decreased levels of mycotoxins *in planta*, DON, D3G and 15-ADON were

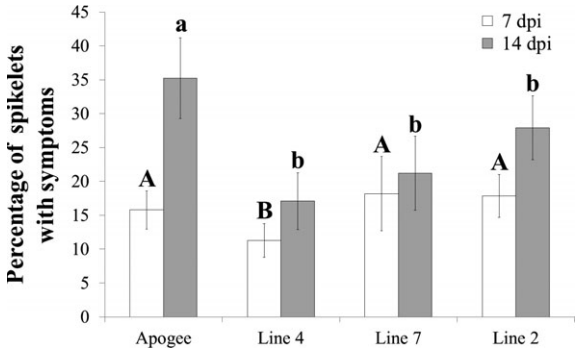

Figure 3 Expression of the *Bradi5g03300* gene increases resistance to initial infection. Percentages of spikelets exhibiting fusarium head blight symptoms on the entire inoculated spikes, 7 days and 14 days after spray inoculation (dpi) by fungal strain FgDON$^+$. Data represent mean values of three independent biological experiments with a minimum of 30 spikes per genotype and per replicate, error bars represent the standard deviation; uppercase and lowercase letters indicate significant differences between Apogee (control line) and each transgenic line at 7 and 14 dpi, respectively; pairwise *t*-tests, $P < 0.05$ ($n > 30$).

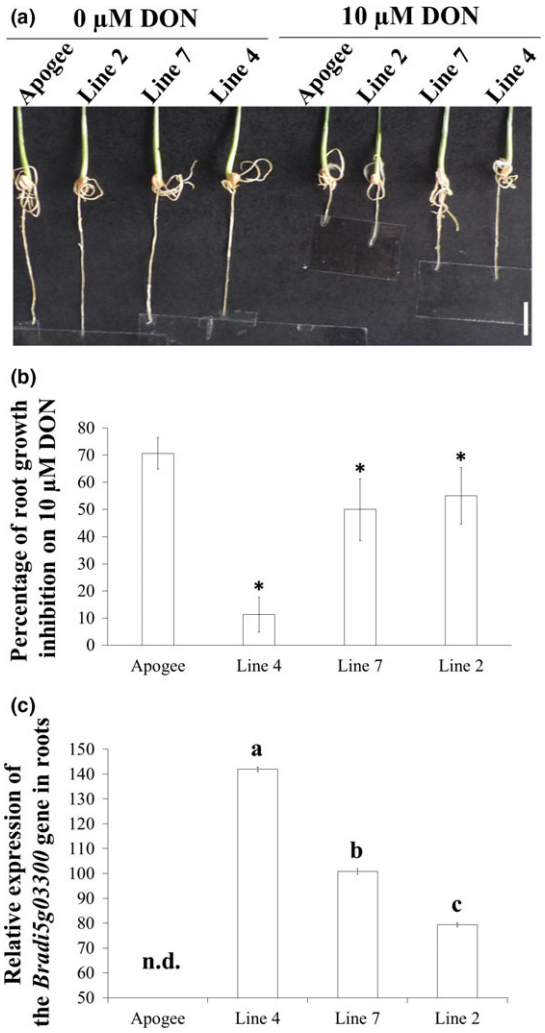

Figure 4 Expression of the *Bradi5g03300* gene is related to root tolerance to DON. (a) Photograph showing typical developmental of the primary seminal root on 7-day-old seedlings of Apogee (control) and each transgenic line on agar medium with or without 10 µM DON. Bar = 1 cm. (b) Percentage of root growth inhibition by 10 µM DON measured on 7-day-old seedlings. Data represent mean values of four independent biological experiments with a minimum of 30 plantlets per genotype and per replicate; error bars represent the standard error, asterisks indicate significant differences between Apogee (control line) and each transgenic line, pairwise *t*-tests, $P < 0.05$ ($n > 30$). (c) Ratio of expression of the *Bradi5g03300* gene in roots of transgenic lines. The relative quantity of gene transcripts was calculated using the following formula: C_t(transgene)/C_t(control gene) × 100. The wheat *ACT* gene was used as endogenous control to normalize the data for differences in input RNA between different samples. Data represent mean values of three independent biological experiments, error bars represent the standard deviation. Different letters indicate significant differences; one-way analysis of variance and Duncan multiple range test ($\alpha = 0.05$). n.d., not determined (nontransformed control). [Colour figure can be viewed at wileyonlinelibrary.com].

quantified in whole spikes of transgenic lines 2, 7 and 4 and control line Apogee at 14 dpi. The quantity of total DON (DON + D3G + 15ADON) was reduced 80%, 55% and 6.4% in lines 4, 7 and 2, respectively, compared to the Apogee control (Fig. 5).

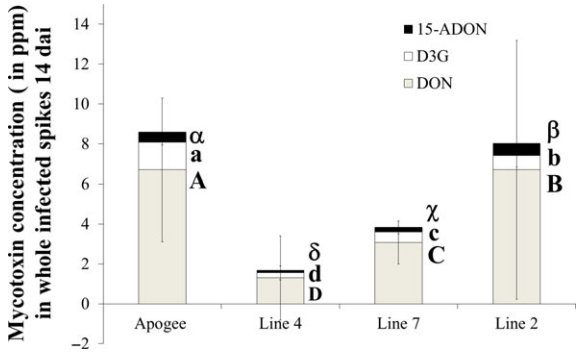

Figure 5 Expression of the *Bradi5g03300* gene reduces total DON content in infected spikes. DON (grey), DON-3-*O*-glucose (D3G) (white) and 15-ADON (black) absolute quantification in whole spikes 14 days after fungal infection. Data represent mean values of three independent biological experiments, error bars represent the standard deviation. Different letters indicate significant differences: DON, uppercase letters; D3G, lowercase letters; 15-ADON, Greek letters. One-way analysis of variance and Duncan multiple range test ($\alpha = 0.01$). [Colour figure can be viewed at wileyonlinelibrary.com].

Discussion

Resistance breeding against FHB aims to obtain both the least grain damage and the least mycotoxin accumulation. Wheat lines able to efficiently convert DON into less toxic masked D3G represent a good strategy in an FHB resistance breeding programme. UDP-glucosyltransferases mainly belonging to family 1 of plant UGTs are able to glucosylate organic compounds and have been shown to be involved in detoxification processes, producing less toxic and more polar molecules, therefore preventing their free diffusion across the lipid bilayer or intracellular compartments (Gachon *et al.*, 2005). Different approaches have been used to identify plant UGTs able to glucosylate DON into D3G. High-throughput screening of *Arabidopsis* UGTs in yeast has shown that UGT73C5 confers resistance to DON by catalysing the formation of D3G (Poppenberger *et al.*, 2003). Screening of cereal UGTs in the same DON-sensitive yeast strain identified UDP-glycosyltransferases able to convert DON into D3G (Schweiger *et al.*, 2010, 2013a,b). Transcriptomic studies identified UGT-encoding genes among strongly induced genes following either *F. graminearum* infection or DON application. Nevertheless, only a few studies have conducted functional analyses *in planta* and even fewer in a host plant for *F. graminearum*, the main causal agent of FHB (Li *et al.*, 2015; Pasquet *et al.*, 2016; Xing *et al.*, 2018; Zhao *et al.*, 2018).

Two major types of resistance to FHB have been reported in wheat. Type I is correlated to the initial infection and type II refers to the spread of FHB symptoms along the infected spikes (Boutigny *et al.*, 2008). To date, DON detoxification has mainly been correlated

with type II resistance. Lemmens *et al.* (2005) have associated DON resistance, through the metabolization of DON into D3G, with resistance to spread of FHB, namely type II resistance. Further reinforcing the correlation, a *F. graminearum* mutant strain unable to produce DON was shown to be able to initiate infection but not to progress along the spike (Maier *et al.*, 2006). Because of the complexity of the corresponding pathogenicity assays and their scoring, type I resistance has been poorly investigated and few studies have been conducted to determine the role of mycotoxin production in the early steps of infection (Boenisch & Schäfer, 2011). Pasquet *et al.* (2016) showed that in *B. distachyon*, increased DON glucosylation *in planta* led to strong type I resistance, suggesting a role for DON in the very early steps of infection.

In the present study, the phenotypic analyses conducted on transgenic wheat lines constitutively expressing the *Bradi5g03300* gene showed that they all exhibit type II resistance to FHB, confirmed by a reduction in fungal biomass, as previously reported in the *B. distachyon* lines constitutively expressing the *Bradi5g03300* gene (Pasquet *et al.*, 2016). Examination of the development of a GFP-expressing strain showed that the difference predominantly resulted from a reduced fungal growth through the rachis. These phenotypes were positively correlated with the level of *Bradi5g03300* expression. An impact on initial infection by *F. graminearum* could also be detected, although much weaker than the one previously observed in *B. distachyon* (Pasquet *et al.*, 2016). This small cereal species has emerged as a good model to study cereal diseases because it can be infected by the same pathogens as small-grain cereal crops and in particular wheat (Opanowicz *et al.*, 2008). Results from this study and Pasquet *et al.* (2016) tend to underline an impact of the plant species on the establishment of type I resistance. This may be due to differences in spike architecture. This could also result from variations in cell wall composition, before or after infection, in these species. Indeed, variations in cell wall composition during *F. graminearum* infection have been already described between susceptible and resistant wheat cultivars (Lahlali *et al.*, 2016). A final explanation could be the pattern of anther extrusion of the wheat variety used in this study, Apogee. Anthers have been shown to constitute a major entry point for initial fungal infection (Miller *et al.*, 2004). The Apogee variety has been proposed as a suitable recipient background to study FHB resistance due to its fast growth, small size and to its amenability to genetic transformation (Mackintosh *et al.*, 2006). However, this variety exhibits a peculiar pattern at mid-anthesis, with anthers blocked between palea and lemma and only partial anther extrusion (Fig. S5), which may alter initial infection following spray inoculation.

Masuda *et al.* (2007) previously reported that DON could inhibit root elongation in *Arabidopsis* and wheat plants. The present work has shown that expression of the *Bradi5g03300* gene in the wheat variety Apogee conferred root tolerance to DON as previously observed in *B. distachyon* (Pasquet *et al.*, 2016). As observed for FHB resistance, DON tolerance was correlated with the level of transgene expression.

In addition to the frequently mentioned type I and type II resistance, one additional category of resistance to FHB that could be used in wheat breeding is type III, referring to DON accumulation (Boutigny *et al.*, 2008). Although metabolization of DON into D3G is not a trait introduced by breeding (Lemmens *et al.*, 2016; Gunupuru *et al.*, 2017), increased levels of D3G in the infected spikes are considered as a resistance-related trait. Lemmens *et al.* (2016) explored whether breeding for FHB resistance resulted in wheat varieties containing higher amounts of the masked mycotoxin, D3G. They concluded that FHB resistant varieties are rather characterized by a reduction of both DON and D3G levels in grain, with a higher impact on DON than on its glucoside. The expression of the barley UGT Hv13248 in wheat showed a rapid and efficient conjugation of DON into D3G (Li *et al.*, 2015). Pasquet *et al.* (2016) showed that *B. distachyon* lines overexpressing the *Bradi5g03300* gene showed increased glucosylation of DON into D3G at early time points but also a reduction of total DON content in late infection stages. In the present study, expression of the *Bradi5g03300* gene in Apogee led to a strong decrease of DON (DON + D3G + 15ADON) content in infected spikes, and the reduction positively correlated with the level of transgene expression, with an 80% decrease towards the control line in the transgenic line exhibiting the higher level of transgene expression. However, an increase in D3G production as compared with the Apogee control line could not be observed. Similar results have already been observed for late infection time points in *B. distachyon* (Pasquet *et al.*, 2016). Indeed, D3G increase in *B. distachyon* transgenic lines overexpressing the *Bradi5g03300* gene was much clearer at early steps of infection (48 hpi, see Pasquet *et al.*, 2016). Further analyses in early stages of infection on wheat transgenic lines expressing the gene will be necessary to better elucidate the timing of DON glucosylation by the Bradi5g03300 UGT as well as the stability of D3G with time in wheat.

Transgenic wheat expressing *Bradi5g03300* did not develop any developmental alteration and set normal viable seeds. However, a slight diminution in seed weight was shown in transgenic lines 4 and 7 compared to the control, and positively correlated with the expression of the transgene. Hence, the increase of the transgene expression may have a slight fitness cost for the plant. In previous work, transgenic wheat lines overexpressing the barley UGT HvUGT13248 did not exhibit altered plant height, with the exception of one line, a phenotype that probably resulted from the insertion locus of the transgene (Li *et al.*, 2015). In *Arabidopsis thaliana*, plants overexpressing the DON-conjugating UGT73C5 have a dwarf phenotype (Poppenberger *et al.*, 2005). This phenotype has been explained by the implication of this UGT in an endogenous function, the glucosylation of brassinosteroids (Poppenberger *et al.*, 2005). The total absence of a

dwarf phenotype obtained with the overexpression of the UGT gene *Bradi5g03300* in Apogee (this study) and also in *B. distachyon* (Pasquet *et al.*, 2016) led to the hypothesis that, apart from its role in DON detoxification, the UGT does not seem to play any endogenous role.

It is concluded that expression of the *Bradi5g03300* UGT-encoding gene in wheat results in a decrease in FHB severity in correlation with a reduction in fungal biomass and the establishment of a strong type II resistance, an increased tolerance to the mycotoxin DON and a reduction of total DON content in infected spikes. Contrary to previous results obtained in *B. distachyon* with the same UGT gene, no impact on resistance to initial infection could be observed, suggesting the existence of species- or genotype-specific traits leading to the establishment of type I resistance to FHB. In a recent study, the same UGT has been shown to glucosylate other trichothecenes, including T-2 and HT-2, even more efficiently than DON (Michlmayr *et al.*, 2018). Future studies on these aspects will be of interest to better deploy detoxification of *Fusarium* sp. mycotoxins as a source of resistance to FHB.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Figure S1. Construction used in biolistics experiments to generate the transforming DNA.

Figure S2. Segregation analyses used to identify homozygous lines. Transgene expression (a) and *PMI* selection gene expression (b) from left to right on cDNA from leaves of plant lines 1–7. Negative controls: water and genomic DNA of nontransformed Apogee. Positive control: mixture (50:50) of genomic DNA of the control line Apogee + cDNA *Bradi5g03300* and cDNA *Bradi5g03300* used during the precedent Gateway multisite strategy. To avoid amplification due to potential gDNA contamination, total RNA was treated with RNase-free DNase prior to reverse transcription (see Materials and methods). Primers 33007-F and 33006-R were used to amplify the gene *Bradi5g03300* leading to a 138 bp amplification product. Primers PMI-F and PMI-R were used to amplify the *PMI* selection gene resulting in a 540 bp PCR product.

Figure S3. Phenotypes of the transgenic lines as compared with the wildtype variety Apogee. (a) Plant height 6, 27 and 39 days after sowing; (b) tiller number 14, 27 and 39 days after sowing; (c) number of spikelets per spike 56 days after sowing. Data represent mean values of three independent experiments with a minimum of 20 plants per replicate, error bars represent the standard deviation. No differences were observed between Apogee (control line) and transgenic lines, pairwise *t*-tests, $P < 0.05$; (d) thousand-grain weight. Data represent mean values of three independent biological experiments, error bars represent the standard deviation. Different letters indicate significant differences; one-way analysis of variance and Duncan multiple range test ($\alpha = 0.05$).

Figure S4. Relative expression of the *Bradi5g03300* gene in leaves of the wheat transgenic lines. The relative quantity of gene transcripts was calculated using: C_t(transgene)/C_t(control gene) × 100. The wheat *ACT* gene was used as endogenous control to normalize the data for differences in input RNA between different samples. Data represent mean values of three independent biological experiments, error bars represent the standard deviation. Different letters indicate significant differences; one-way analysis of variance and Duncan multiple range test ($\alpha = 0.05$).

Figure S5. Typical appearance of an Apogee spike at mid-anthesis. The photograph was taken 56 days after sowing (BBCH65 stage). Arrows indicate semi-extrusive anthers. Bar 5 mm.

Table S1. List of primers used in RT-qPCR and qPCR experiments.