Transgenic Wheat Expressing a Barley UDP-Glucosyltransferase Detoxifies Deoxynivalenol and Provides High Levels of Resistance to *Fusarium graminearum*

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Fusarium head blight (FHB), mainly caused by *Fusarium graminearum*, is a devastating disease of wheat that results in economic losses worldwide. During infection, *F. graminearum* produces trichothecene mycotoxins, including deoxynivalenol (DON), that increase fungal virulence and reduce grain quality. Transgenic wheat expressing a barley UDP-glucosyltransferase (*HvUGT13248*) were developed and evaluated for FHB resistance, DON accumulation, and the ability to metabolize DON to the less toxic DON-3-*O*-glucoside (D3G). Point-inoculation tests in the greenhouse showed that transgenic wheat carrying *HvUGT13248* exhibited significantly higher resistance to disease spread in the spike (type II resistance) compared with nontransformed controls. Two transgenic events displayed complete suppression of disease spread in the spikes. Expression of *HvUGT13248* in transgenic wheat rapidly and efficiently conjugated DON to D3G, suggesting that the enzymatic rate of DON detoxification translates to type II resistance. Under field conditions, FHB severity was variable; nonetheless, transgenic events showed significantly less-severe disease phenotypes compared with the nontransformed controls. In addition, a seedling assay demonstrated that the transformed plants had a higher tolerance to DON-inhibited root growth than nontransformed plants. These results demonstrate the utility of detoxifying DON as a FHB control strategy in wheat.

Fusarium head blight (FHB) (scab), primarily caused by *Fusarium graminearum*, is a disease of wheat and barley that results in dramatic losses of grain yield and quality (Goswami and Kistler 2004; Kazan et al. 2012; Parry et al. 1995). During

infection, *F. graminearum* produces trichothecene mycotoxins, including deoxynivalenol (DON), which facilitate disease development and contaminate grain (Goswami and Kistler 2004). Grain products contaminated with trichothecenes pose human and animal health issues when consumed (Pestka 2010; Bin-Umer et al. 2011). For this reason, the United States Food and Drug Administration has set the advisory level of DON at 1 ppm (mg kg⁻¹) in finished wheat products for human consumption (United States Food and Drug Administration 2010). Similarly, the European Union has established a maximum tolerance level of 0.75 ppm for DON in food commodities for human consumption (European Commission 2006). Therefore, reducing trichothecene concentrations in grains is essential both for food safety and in disrupting FHB development.

The two major types of FHB resistance in wheat are recognized as type I (resistance to initial infection) and type II (resistance to disease spread in infected spikes) (Mesterhazy 1995). Numerous quantitative trait loci (QTL) associated with type I and II resistance have been identified (Anderson et al. 2001; Buerstmayr et al. 2009; Waldron et al. 1999). A major QTL for type II resistance was identified in wheat on the short arm of chromosome 3B and is referred to as *Fhb1* (Anderson et al. 2001; Liu et al. 2006; Waldron et al. 1999). Intensive breeding efforts have been established that have resulted in incorporation of QTL into elite breeding lines; however, the level of resistance conferred by these QTL is insufficient to alone provide protection to FHB, and the mechanisms of resistance provided by these QTL are not clear (Buerstmayr et al. 2009). Transgenic wheat events expressing host defense genes and DON-resistant genes have been developed, but these have only resulted in modest reductions in disease severity (Di et al. 2010; Mackintosh et al. 2007; Okubara et al. 2002; Shin et al. 2008). Although a recent report on host-induced gene silencing of a chitin synthase gene in transgenic wheat showed high levels of resistance to FHB (Cheng et al. 2015), there is a need to explore additional genetic mechanisms that perturb the infection process, thus resulting in improved FHB resistance.

Previous studies have shown that trichothecenes act as virulence factors during FHB disease development by contributing to disease spread in the infected spikes. *F. graminearum* strains carrying loss-of-function mutations in the *TRI5* gene, which encodes the first enzyme in the trichothecene biosynthetic pathway, resulted in the lack of trichothecene production and reduced virulence on wheat (Proctor et al. 1995). Noteworthy, these trichothecene-deficient

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strains of *F. graminearum* can infect wheat but were unable to spread within the spike, demonstrating that trichothecenes are not required for initial infection but that they do play an important role in the spread of disease symptoms in the spike (Bai et al. 2002; Desjardins et al. 1996; Jansen et al. 2005; Proctor et al. 1997). Additional studies on the infection strategy of *Fusarium* spp. revealed that the pathogen has a biotrophic growth phase for about 48 h after spore germination. Subsequently, the pathogen produces trichothecenes and transitions to a necrotrophic strategy (Brown et al. 2010; Kazan et al. 2012; Lysøe et al. 2011). These findings indicate that suppression of trichothecene accumulation at early time points may be essential for limiting FHB development. Previous work by Lemmens et al. (2005) examined a population segregating for *Fhb1* QTL and found that lines containing the resistant *Fhb1* allele exhibited an increased ability to conjugate DON with glucose to the less-toxic DON-3-*O*-glucoside (D3G). These results indicated that the conjugation of DON to D3G is a potential resistance mechanism, and the authors proposed that *Fhb1* likely encodes for either a UDP-glucosyltransferase (UGT) or a gene that regulates UGT activity. Taken together, this information suggests that trichothecenes play a major role in pathogen virulence and the identification of appropriate genes that encode for UGTs for subsequent expression in transgenic plants may serve as an avenue to reduce DON accumulation and, in turn, disease severity.

Large gene families encode UGTs in plants (Caputi et al. 2012; Ross et al. 2001). These genes function by glucosylating plant metabolites and can also act to detoxify virulence factors produced by pathogens. An *Arabidopsis* UGT (*DOG1* or *AtUGT73C5*) was shown to conjugate DON at the carbon 3 position (C3-OH) group with glucose to form D3G (Poppenberger et al. 2003). Overexpression of *AtUGT73C5* in transgenic *Arabidopsis* resulted in DON resistance (Poppenberger et al. 2003). However, these same transgenic plants exhibited a dwarf phenotype, likely due to the conjugation of brassinosteroid

brassinolide to the inactive brassinolide-23-*O*-glucoside (Poppenberger et al. 2005). A barley UGT, *HvUGT13248*, is up-regulated by *F. graminearum* infection and DON application (Boddu et al. 2007; Gardiner et al. 2010). Functional characterization of *HvUGT13248* showed that expression in both yeast and *Arabidopsis* imparts DON resistance by converting DON to D3G (Schweiger et al. 2010; Shin et al. 2012). In contrast to the transgenic *Arabidopsis* expressing *DOG1*, *Arabidopsis* plants expressing *HvUGT13248* did not exhibit a dwarf phenotype and the conjugation of brassinosteroids was not observed (Shin et al. 2012). Moreover, *Brachypodium distachyon* possesses two UGT homologs to *HvUGT13248*, which also showed resistance to high levels of DON by formation of D3G in yeast (Schweiger et al. 2013). These results suggest UGTs are promising genes for increased FHB resistance in wheat.

The overall goals of this study were to develop transgenic wheat expressing *HvUGT13248* and to examine whether this gene increases resistance to *F. graminearum* infection and DON. The specific objectives of this study were i) to develop transgenic wheat constitutively expressing *HvUGT13248*, ii) to evaluate these wheat lines for resistance to FHB in the greenhouse and in field trials, iii) to assess these wheat lines for resistance to DON, and iv) to monitor the conversion of DON to D3G in planta.

RESULTS

Generation of transgenic wheat expressing *HvUGT13248*.

The plasmid, designated pZP212-*HvUGT13248*, carrying the *HvUGT13248* gene driven by the maize ubiquitin promoter, coupled with its first intron (Christensen et al. 1992) (Fig. 1A) was used for particle bombardment transformation of the wheat cv. Bobwhite (BW) and *Agrobacterium*-mediated transformation of the wheat cv. CBO37. The *HvUGT13248* open reading frame has a C-terminal FLAG epitope, which enables

Fig. 1. Transformation plasmid and characterization of transgenic wheat. **A**, The pZP212 plasmid containing the *HvUGT13248* transgene (pZP212-*HvUGT13248*) was used for wheat transformation. *NPT II* = neomycin phosphotransferase II gene; Ubi-1 pro. = maize ubiquitin-1 promoter with the first intron; 35S pro. = *Cauliflower mosaic virus* 35S promoter; FLAG = FLAG-epitope tag for Western blotting. **B**, Southern blot analysis using ³²P-dCTP labeled *NPTII* gene and *Xba*I-digested genomic DNA. **C**, Western blot analysis using FLAG-epitope antibody. Events #8, #14, #15, #19, #34, and #37 were independent transgenic lines in the Bobwhite (BW) background and BW was the nontransformed control; #1381, #1726, #1386, and #1383 were independent transgenic lines in the CBO37 background and CBO37 was the nontransformed control.

tracking of the *HvUGT13248* protein with FLAG-epitope antibodies. To this end, Western blot analysis using the FLAG-epitope antibodies was conducted on the T₁ progeny derived from the primary transgenic events. Six BW transgenic events (#8, #14, #15, #19, #34, and #37) and four CBO37 transgenic events (#1381, #1726, #1386, and #1383) accumulating detectable *HvUGT13248*-FLAG protein levels were selected and were allowed to self-pollinate (Fig. 1C). Variation in expression levels of the transgene was observed across the selected events, with the nontransformed BW and CBO37 controls displaying no detectable signal (Fig. 1C). Coexpression of the *HvUGT13248* protein with the NPTII selectable marker (enzyme-linked immunosorbent assay [ELISA] assay, described below) was observed. Thus, the NPTII ELISA assay was a convenient and reliable marker for the presence of the active *HvUGT13248* transgene and subsequent determinations of *HvUGT13248* transgene expression were based on the NPTII ELISA assay.

A Southern blot analysis was conducted on T₃ individuals from the selected transgenic events to determine if they originated from independent transformation events. Genomic DNA was digested with the *Xba*I restriction endonuclease and was probed with the full-length NPTII gene. The probe did not hybridize to BW or CBO37 nontransformed controls, while each of the BW-derived and the CBO37-derived transgenic events exhibited different banding patterns, demonstrating that the transgene was successfully integrated into these lines and that they originated from independent transformation events (Fig. 1B).

Evaluation of type II resistance conferred by transgenic wheat expressing *HvUGT13248*.

To evaluate type II resistance in the transgenic wheat events, we performed point inoculation experiments under greenhouse conditions. For each screen, except for the Spring 2011 trial when there was limited seed availability, 20 plants of each transgenic event were grown and ELISA for the NPTII protein was performed on each plant. Between two to 20 plants from each transgenic event expressed the transgene, indicating that expression was relatively stable over generations (Supplementary Table S1). Plants expressing *HvUGT13248* and, in some cases, the nonexpressing siblings (UGT-) were screened for FHB resistance. The susceptible check cv. Wheaton exhibited 84.7% FHB severity on average across all three trials, while the resistant check cv. Sumai 3 exhibited an average FHB severity of 10.1%, indicating that the environment for disease progression was successful and discriminative. Our results show the transgenic events significantly reduced FHB severity compared with the nonexpressing siblings and nontransformed BW and CBO37 controls (Fig. 2). Compared with the nontransformed BW control, the reduction of disease severity across screening T₁, T₂, and T₃ plants from the BW transgenic events was at least 74%. Moreover, events #15 and #19 displayed a high degree of type II resistance in the greenhouse assays, with negligible fungal spread within the spike, an equivalent type II resistant phenotype to the Sumai 3 control. In a similar fashion, the combined results from the FHB greenhouse tests of the CBO37-derived transgenic events #1381, #1726, #1386, and #1383 displayed significant reduction in disease severity greater than 64% (Fig. 2), with CBO37 showing a more susceptible FHB phenotype than BW. Taken together, our results revealed that expression of *HvUGT13248* in wheat translates to enhanced type II resistance.

Transgenic wheat expressing *HvUGT13248* promotes DON to D3G conversion.

To determine if the expression of *HvUGT13248* in transgenic wheat is catalyzing the conjugation of DON to D3G, hence contributing to the observed type II resistance, we monitored

the rate of DON conjugation via quantification of DON and D3G over time. To this end, direct DON-challenge assays on transgenic event #19 and the corresponding BW control were conducted. Plants from event #19 were challenged with DON at a 2 µg per spikelet rate applied to 25 spikelets (50 µg or 169 nmol in total per spike) on the main spike per individual. Tissues samples were taken at 1, 3, 7, 14, and 21 days after DON challenge. DON and D3G levels were ascertained by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). The conversion of DON to D3G was observed in event #19 and the nontransformed BW. However, the conversion of DON to D3G was more rapid in event #19 relative to BW, with an average of 24% DON converted to D3G in event #19 within the first 24 h after DON application, while there was

Fig. 2. *Fusarium* head blight (FHB) greenhouse screen of transgenic wheat expressing *HvUGT13248* at 21 days after point-inoculation. FHB severity was calculated as the percentage of symptomatic spikelets per spike at 21 days after inoculation. Wheaton was the susceptible check; Sumai 3 was the resistant check. **A**, Events #8, #14, #15, #19, #34, and #37 are transgenic wheat expressing *HvUGT13248* in the Bobwhite (BW) background; and **B**, events #1381, #1726, #1386, and #1383 are transgenic wheat expressing *HvUGT13248* in the CBO37 background.

only 2% DON conversion in BW. Between 1 and 3 days after DON application, the conversion of DON to D3G was greater in the nontransformed than transgenic plants, likely due to the lower level of DON in the transgenic plants. By 21 days after challenge, residual DON levels present in the transgenic plants were only 26% of that in BW (Fig. 3A and C). In addition, the D3G/DON ratio trended higher in event #19 compared with BW (Fig. 3B). These results clearly demonstrate in planta UGT activity in the transgenic samples leads to the enhanced conversion of DON to D3G.

Evaluation of field-based resistance conferred by transgenic wheat expressing *HvUGT13248*.

Transgenic wheat expressing *HvUGT13248* exhibited a high level of type II resistance under greenhouse conditions. To gain insight on the translation of the resistance phenotype to a field environment, spray-inoculated experiments in mist-irrigated plots were conducted over the 2012 to 2014 growing seasons. Selected BW-derived transgenic lines were represented each year, while those from CBO37-derived transgenic lines were only in the 2013 and 2014 trials. Data on the various FHB-related phenotypes were monitored, including FHB incidence (percentage of spikes with visually symptomatic spikelets of the 20 heads observed), FHB severity (percentage of symptomatic

spikelets of the total of all spikelets observed), percentage of visually scabby kernels (VSK), and DON concentration in harvested grain (Table 1). In addition, D3G concentration in grain from the 2013 and 2014 field trials was ascertained (Table 1).

Overall, FHB incidence, severity, and VSK in the BW- and CBO37-derived transgenic lines exhibited variable responses compared with the BW and CBO37 nontransformed controls, respectively. All transgenic lines exhibited a statistically significant reduction in FHB incidence and severity in at least one trial except for event #1381 and VSK for at least one trial except for events #14 and #1381. Noteworthy, all BW-derived transgenic plants exhibited a statistically significant reduction in FHB severity in all three trials except for event #37 in 2014. Importantly, event #37 in 2012 and 2013 and event #1726 in 2013 and 2014 exhibited similar levels of FHB severity and VSK compared with the type II resistant commercial varieties Rollag and Alsen that carry the *Fhb1* QTL. It is also worth noting that most transgenic events showed significantly reduced FHB incidence except in 2014. Taken together, our results demonstrate that transgenic wheat expressing *HvUGT13248* can reduce the level of FHB severity that is comparable to FHB-resistant commercial varieties and the FHB incidence and VSK can be reduced when FHB disease pressure is moderate (e.g., 2013).

Fig. 3. Conjugation of deoxynivalenol (DON) to DON-3-O-glucoside (D3G) in transgenic wheat expressing *HvUGT13248*. **A**, DON and D3G concentrations in Bobwhite (BW) and transgenic line #19 at each time point, from 1 to 21 days after DON application. **B**, Fold change of molar ratio of D3G to DON concentrations in BW and transgenic line #19 at each time point. **C**, Rate of DON converted to D3G over time.

Since *HvUGT13248* conjugates DON to D3G, the expected outcome is a lower concentration of DON, a higher concentration of D3G, and a higher ratio of D3G/DON in the transgenic plants compared with the nontransformed controls. All transgenic lines except #19 showed a higher ratio of D3G/DON than nontransformed controls in at least one trial; however, we observed several possible combinations of DON and D3G concentration in the transgenic plants compared with the nontransformed controls. Consistent with our initial prediction for just the DON and D3G concentrations were lines #14 and #1386 in 2013, which exhibited statistically significant decreases in DON concentration and significant increases in D3G concentration in the transgenic plants compared with the nontransformed controls. We also observed significant decreases in DON and nonsignificant changes in D3G concentration (e.g., lines #8, #15, and #19 in 2013; lines #1726 and #1383 in 2014), significant decreases in both DON and D3G (e.g., line #1726 in 2013), nonsignificant changes in DON concentration and

significant increases in D3G concentration (e.g., lines #8, #14, #15, and #37 in 2014), and nonsignificant changes in DON and D3G concentration (e.g., #1383 in 2013; #19 in 2014, #1381 in 2013 and 2014). These results show that *HvUGT13248* conjugates DON to D3G in field conditions but that there are considerable environmental factors that play a role in the DON and D3G concentrations in the grain.

The three field trials provided the opportunity to assess the protection *HvUGT13248* offered in environments exhibiting different levels of FHB pressure and to assess the relationship between the ratio of DON and D3G concentration and disease severity. We judged the severity of each field trial based on the level of FHB incidence in the check lines included each year. In the low disease environment in 2013, the transgenic plants exhibited the lowest level of incidence, severity, and VSK. Noteworthy, in 2013, the DON concentrations of all the transgenic plants were lower than the controls, and, in the BW-derived transgenic events, the DON and D3G combined levels

Table 1. Field screening results of transgenic wheat expressing *HvUGT13248*^a

Line	Incidence ^b	Severity ^c	VSK ^d	DON ^e	D3G ^e	D3G/DON	D3G+DON ^e
2012							
BW	96 ± 2	40 ± 4	26 ± 5	38 ± 5	NA	NA	NA
#8	76 ± 7*	15 ± 8**	18 ± 1	55 ± 4*	NA	NA	NA
#14	89 ± 8	24 ± 2**	20 ± 4	40 ± 8	NA	NA	NA
#15	72 ± 6**	10 ± 2***	9 ± 1**	27 ± 5	NA	NA	NA
#19	65 ± 9**	11 ± 2***	14 ± 1*	28 ± 2	NA	NA	NA
#37	56 ± 9**	7 ± 2***	9 ± 2***	26 ± 2*	NA	NA	NA
Rollag	91 ± 5	27 ± 3	16 ± 3	18 ± 3	NA	NA	NA
Sumai 3	48 ± 1	4 ± 0	4 ± 1	8 ± 3	NA	NA	NA
Alsen	90 ± 5	25 ± 3	16 ± 2	3 ± 7	NA	NA	NA
Wheaton	98 ± 1	42 ± 7	75 ± 0	59 ± 12	NA	NA	NA
2013							
BW	91 ± 4	27 ± 5	8 ± 1	26 ± 3	8 ± 1	0.30 ± 0.02	34 ± 4
#8	74 ± 6*	11 ± 2*	5 ± 1**	12 ± 2**	15 ± 4	1.16 ± 0.23*	27 ± 6
#14	81 ± 2*	12 ± 1*	7 ± 1	18 ± 2*	17 ± 1**	0.94 ± 0.13**	35 ± 2
#15	68 ± 6*	9 ± 1**	3 ± 1***	8 ± 1***	8 ± 3	0.95 ± 0.34	16 ± 4*
#19	63 ± 4**	11 ± 2*	5 ± 0**	11 ± 3**	7 ± 3	0.52 ± 0.14	18 ± 6
#37	39 ± 8***	4 ± 1**	4 ± 1**	11 ± 2**	13 ± 5	1.06 ± 0.31	24 ± 6
CBO37	83 ± 3	17 ± 3	19 ± 1	39 ± 3	22 ± 5	0.57 ± 0.12	61 ± 6
#1381	76 ± 7	15 ± 4	16 ± 1	30 ± 4	35 ± 7	1.13 ± 0.11*	65 ± 11
#1726	44 ± 9**	5 ± 1**	4 ± 1***	8 ± 1***	6 ± 1*	0.71 ± 0.09	14 ± 2***
#1386	55 ± 5**	7 ± 1**	11 ± 2**	27 ± 4*	56 ± 9**	2.07 ± 0.12***	83 ± 13
#1383	74 ± 4	9 ± 2*	8 ± 2***	28 ± 5	33 ± 7	1.16 ± 0.08**	61 ± 11
Rollag	63 ± 11	7 ± 1	5 ± 1	10 ± 1	3 ± 0	0.35 ± 0.05	14 ± 0
Sumai 3	44 ± 3	3 ± 0	2 ± 1	7 ± 2	2 ± 1	0.46 ± 0.21	10 ± 3
Alsen	75 ± 5	11 ± 1	5 ± 1	8 ± 1	2 ± 1	0.29 ± 0.10	10 ± 1
Wheaton	95 ± 3	34 ± 4	43 ± 1	29 ± 5	7 ± 2	0.22 ± 0.04	35 ± 7
2014							
BW	100 ± 0	64 ± 3	22 ± 2	74 ± 16	6 ± 1	0.09 ± 0.02	81 ± 17
#8	100 ± 0	33 ± 3***	18 ± 2	107 ± 16	21 ± 1***	0.22 ± 0.04*	128 ± 16
#14	98 ± 3	42 ± 6**	21 ± 2	95 ± 14	21 ± 3**	0.23 ± 0.03**	115 ± 16
#15	96 ± 2	37 ± 11*	21 ± 3	75 ± 12	16 ± 2**	0.22 ± 0.02**	91 ± 14
#19	100 ± 0	47 ± 4**	24 ± 4	77 ± 10	10 ± 1	0.13 ± 0.01	87 ± 11
#37	99 ± 1	57 ± 4	20 ± 3	64 ± 9	19 ± 2**	0.30 ± 0.02***	83 ± 12
CBO37	98 ± 1	34 ± 5	25 ± 3	111 ± 8	17 ± 2	0.16 ± 0.02	128 ± 9
#1381	91 ± 3	25 ± 4	19 ± 1	84 ± 12	20 ± 3	0.23 ± 0.01**	104 ± 14
#1726	84 ± 7	16 ± 2**	16 ± 3*	52 ± 8**	18 ± 1	0.38 ± 0.08*	70 ± 7**
#1386	95 ± 2	32 ± 3	20 ± 1	98 ± 21	25 ± 1*	0.28 ± 0.05	123 ± 20
#1383	81 ± 6*	13 ± 1**	8 ± 1**	57 ± 6**	16 ± 1	0.28 ± 0.03**	73 ± 7**
Rollag	93 ± 1	23 ± 1	15 ± 2	32 ± 8	2 ± 0	0.07 ± 0.01	34 ± 8
Sumai 3	91 ± 2	18 ± 2	9 ± 2	22 ± 4	3 ± 0	0.13 ± 0.02	25 ± 4
Alsen	93 ± 3	29 ± 3	18 ± 4	42 ± 9	4 ± 0	0.10 ± 0.03	46 ± 9
Wheaton	98 ± 3	72 ± 9	75 ± 0	107 ± 21	6 ± 0	0.07 ± 0.01	113 ± 21

^a Events #8, #14, #15, #19 and #37 are transgenic wheat expressing *HvUGT13248* in the Bobwhite (BW) background, and events #1381, #1726, #1386 and #1383 are expressing *HvUGT13248* in the CBO37 background. Wheaton was the susceptible check; Alsen, Rollag and Sumai 3 were type II resistant checks; BW and CBO37 were the nontransformed controls. Values provided are the means ± standard error. *, ** and *** indicate significance at the 0.05, 0.01 and 0.001 levels compared with the BW or CBO37 nontransformed controls. NA = not available.

^b *Fusarium* head blight (FHB) incidence, the percentage of spikes with visually symptomatic spikelets of the 20 heads observed.

^c FHB severity, the percentage of diseased spikelets of the total of the 20 spikes observed.

^d VSK (%): the percentage of visually scabby kernels (VSK).

^e Deoxynivalenol (DON), DON-3-O-glucoside (D3G), and D3G+DON concentrations are shown in nanomoles per gram.

were lower than those of the nontransformed BW lines, except for transgenic event #14. In the higher disease environment in 2012, we observed a trend toward a significant reduction in FHB incidence, FHB severity, and VSK, but the DON concentration in the transgenic plants compared with the control was not significantly different. In the highest disease environment in 2014, we observed a significant decrease in FHB severity of the transgenic plants, although the other disease parameters exhibited high variation. Only transgenic line #1726 showed consistently high levels of resistance across the disease environments, with lower FHB incidence, a higher D3G/DON ratio, and statistically significantly lower FHB severity, VSK, DON, and DON and D3G combined than the nontransformed CBO37. Our results indicate a general trend for the transgenic plants to perform best in an environment with reduced disease pressure, although, even under high disease pressure, the transgenic plants can confer a significant reduction in FHB incidence, FHB severity, and VSK, but considerable variability with regards to DON and D3G concentration is apparent.

Transgenic wheat expressing *HvUGT13248* provides resistance in roots to DON.

DON is known to inhibit root elongation in *Arabidopsis* and wheat (Masuda et al. 2007), so we examined the root growth of selected *HvUGT13248* events on DON-supplemented growth medium (Fig. 4, Supplementary Figs. S1 and S2). T₄ seeds of homozygous lineages derived from transgenic events #8, #15, and #37 along with the corresponding control BW were germinated on Murashige and Skoog (MS) medium for 24 h and were subsequently transferred to MS medium supplemented with 0, 2, 5, and 10 ppm (milligrams per liter) DON. Root growth was measured once a day from 3 to 7 days after germination. In control treatments (0 ppm DON), root-growth measurements for the transgenic events were not significantly different from BW (Fig. 4). However, in plates containing DON, root growth from the transgenic events was significantly longer than BW (Fig. 4). Similar results were observed in the transgenic events in the CBO37 background.

Fig. 4. Root growth of transgenic wheat lines (#8, #15, and #37) in the Bobwhite background expressing *HvUGT13248* on Murashige and Skoog medium containing 0, 2, 5, and 10 ppm deoxynivalenol (DON).

Expression of *HvUGT13248* in wheat does not alter morphology.

To determine if the expression of *HvUGT13248* in wheat resulted in morphological changes compared with the controls, we measured the height of the transgenic wheat plants and the controls in field trials in 2013 and 2014. Our results showed that the transgenic plants were indistinguishable, except for #1726 in 2013, from the controls under both field environments (Supplementary Fig. S3).

DISCUSSION

Transgenic wheat expressing *HvUGT13248* exhibit FHB resistance and does not alter morphology.

Plant UGTs have previously been demonstrated to conjugate DON to the less toxic metabolite D3G in both yeast (Poppenberger et al. 2003; Schweiger et al. 2010, 2013) and *Arabidopsis* (Poppenberger et al. 2003; Shin et al. 2012) systems. To address the potential to translate these findings from model systems to commodity crops as a means to mitigate pathogenesis of *Fusarium* spp., we introduced *HvUGT13248* into two wheat genetic backgrounds, BW and CBO37, and subsequently phenotyped a set of derived transgenic events under laboratory, greenhouse, and field-based environments. The data tabulated from these studies clearly show that transgenic wheat events expressing *HvUGT13248* had significantly lower FHB severities compared with their respective controls, over diversified environmental conditions and across two genetic backgrounds (Table 1; Fig. 2). The results herein reveal *HvUGT13248* expression in wheat can lead to an equivalently high level of type II resistance to the type II-resistant cv. Sumai 3 (Fig. 2). Previous transgenic approaches to enhance FHB resistance in wheat included the introduction of antifungal proteins wherein reduction in disease severity was modest in the greenhouse, ranging from 30 to 52% (Di et al. 2010; Mackintosh et al. 2007; Shin et al. 2008). Importantly, *HvUGT13248* not only enhances the type II resistance response but also led to significant reduction in FHB incidence, except in 2014, when disease pressure was high, which indicates that *HvUGT13248* may also contribute to the type I resistance response by detoxifying DON at an early stage of initial infection. Interestingly, trichothecene biosynthesis is induced during the establishment of initial infection (Boenisch and Schäfer, 2011) but not needed for initial infection (Bai et al. 2002). Thus, additional research is required to determine the role that conjugation of DON to D3G may play in resistance to initial infection.

HvUGT13248 belongs to the glycosyltransferase gene superfamily, which includes a plethora of homologous genes in plants, including *Arabidopsis thaliana*, *Brachypodium distachyon*, wheat, and barley (Caputi et al. 2012; Li et al. 2001; Schweiger et al. 2010, 2013). The *Arabidopsis DOGT1* gene is capable of conjugating DON to D3G, and overexpression of *DOGT1* in *Arabidopsis* resulted in increased tolerance against DON and 15-ADON (15-acetylated DON) (Poppenberger et al. 2003). However, *DOGT1* also catalyzes glucoside conjugation with brassinosteroids, resulting in a dwarf phenotype in transgenic *Arabidopsis* plants (Poppenberger et al. 2005). Unlike *DOGT1*, *HvUGT13248* does not produce brassinosteroid glucosides in *Arabidopsis* plants, and the morphology of the transgenic *Arabidopsis* was similar to the non-transformed controls (Shin et al. 2012). Consistent with the previous results in *Arabidopsis*, our results showed that expression of *HvUGT13248* in wheat, with the exception of #1726 in 2013, does not alter plant height.

Rapid and large amount of conjugation of DON to D3G results in type II resistance.

The underlying mechanism associated with the observed enhanced type II resistance imparted by expression of the

HvUGT13248 in wheat is enzymatic activity leading to the increased conversion rate of DON to D3G (Fig. 3). Although DON glycosylation is a common detoxification strategy in cereal crops and D3G is identified in naturally contaminated fields (Berthiller et al. 2005, 2009), our results show that the rate and amount of conjugation, especially at the early stage of infection, is important for resistance. In our feeding experiment, BW converted DON to D3G; however, transgenic line #19 converted a larger portion of DON to D3G in the first 24 h. In our field tests, both nontransformed controls BW and CBO37 accumulated higher levels of D3G compared with resistant checks (Sumai 3, Rollag, and Alsen), and even the susceptible check Wheaton showed the ability to convert DON to D3G. However, in all these lines, the ratio of D3G/DON is much lower than all of the transgenic lines. These results are consistent with previous reports of transgenic yeast and *Arabidopsis* expressing *HvUGT13248* that showed higher resistance to DON in the growth media and, ultimately, higher ratios of D3G/DON compared with the nontransformed controls (Schweiger et al. 2010; Shin et al. 2012).

Previous attempts to provide FHB resistance through trichothecene metabolism via a detoxification route were largely ineffective. The *Tri101* gene encodes an enzyme that catalyzes the acetylation of the hydroxyl group at the C3-OH of trichothecene precursors to reduce toxicity (McCormick et al. 1999). Transgenic tobacco plants expressing the *F. sporotrichioides*-derived *Tri101* gene (*FsTri101*) showed increased tolerance to trichothecenes (Muhitch et al. 2000). However, when *FsTri101* was overexpressed in transgenic wheat (Okubara et al. 2002), the derived transgenic plants only showed partial type II resistance after point inoculation under greenhouse conditions.

D3G is considered a 'masked' trichothecene (Berthiller et al. 2005), given that D3G is not monitored during routine assays for trichothecenes but can be converted back to DON by the gut microflora of mammals (Berthiller et al. 2011). Thus, although D3G is less phytotoxic than DON (Poppenberger et al. 2003), the accumulation of D3G in grain is still considered a potential health issue. However, the rapid conjugation of DON to D3G during the early stages of infection may reduce overall fungal load, which in turn may result in reduced DON and D3G in grain. For example, the most resistant transgenic events, #37 and #1726, both accumulated lower total toxin levels (DON+D3G) in the 2013 field trial, as compared with the corresponding nontransformed controls (Table 1).

In summary, our results show that transgenic wheat expressing *HvUGT13248* is an intriguing option for further exploration with the goal to detoxify DON and control FHB.

MATERIALS AND METHODS

Plant materials.

The spring wheat cultivars Alsen, Sumai 3, Rollag, Wheaton, BW, and CBO37 were used for the experiments. BW and CBO37 were used for transformation experiments. Wheaton and CBO37 are spring wheat cultivars that are highly susceptible to FHB. BW is a spring wheat cultivar that exhibits moderate susceptibility. Alsen and Rollag are spring wheat cultivars carrying the *Fhb1* QTL inherited from Sumai 3 and exhibit type II resistance and are moderately resistant. Sumai 3 is a Chinese cultivar that exhibits high levels of type II FHB resistance.

Plant transformation vector and wheat transformation.

The 1,452-bp barley UDP-glucosyltransferase (*HvUGT13248*) gene containing a carboxyl-terminal FLAG-tag sequence was cloned into the pENTER TMD TOPO vector (Shin et al. 2012) and was then inserted into the binary expression plasmid pIPKb002 (Himmelbach et al. 2007), using the Gateway LR recombination

reaction. The Ubi1 promoter forward 5'-GGGAAGCTTGGCCT TACTAGGCTGCAGTG-3' and nopaline synthase (NOS) terminator reverse 5'-CCCGGTACCCGCGTCGAGCGATCTAGTA-3' primers were used for the *HvUGT13248* polymerase chain reaction (PCR) amplification including the Ubi1 promoter and NOS terminator. The 2.9-kb PCR product was digested and cloned into the unique *HindIII* and *KpnI* sites of the vector pZP212. The pZP212 plasmid contains the *NPTII* gene driven by the *Cauliflower mosaic virus* 35S promoter and terminated by the NOS gene from *Agrobacterium tumefaciens* (Hajdukiewicz et al. 1994). The spring wheat cultivar BW was used for transformation by particle gun bombardment method. The transformation protocols, including particle gun bombardment of embryos, tissue culture selection, and plant regeneration, were conducted according to the protocol described by Mackintosh and colleagues (2006). The CBO37-derived transgenic lines were produced using *Agrobacterium*-mediated transformation according to Cheng and colleagues (1997).

Characterization of transgenic wheat expressing *HvUGT13248*.

For Southern blot analysis of transgenic wheat carrying the *HvUGT13248* gene, genomic DNA (10 µg) was digested with endonuclease *XbaI*, was separated on a 1% agarose gel, and was transferred onto Hybond N⁺ membranes (Amersham Biosciences, Piscataway, NJ, U.S.A.). The *NPTII* gene probe (795 bp) was derived from a PCR-amplified product. The forward 5'-ATGATTGAACAAGATGGATTG-3' and reverse 5'-TCAGAAGAACTCGTCAAG-3' primers were used for the *NPTII* gene probe PCR amplification. The probe sequence was labeled with ³²P dCTP using the Prime-a-Gene labeling system (Promega, Madison, WI, U.S.A.), following the manufacturer's instructions. The radiolabeled *NPTII* gene was used as a probe for the hybridization, and the subsequent banding patterns were visualized using autoradiography.

For Western blot analysis, protein was extracted by grinding leaf tissues in extraction buffer (2% sodium dodecyl sulfate [SDS], 60 mM Tris-HCl [pH 6.8], 14.4 mM β-mercaptoethanol, 10% glycerol, and 0.1% [wt/vol] bromophenol blue) and cell debris was removed by centrifugation. Total protein concentration was determined using Bio-Rad reagent (Bio-Rad, Hercules, CA, U.S.A.), with bovine serum albumin as a standard. Protein extracts (10 µg for BW transgenic lines and 15 µg for CBO37 lines) were separated by SDS-polyacrylamide electrophoresis (12% acrylamide) and were transferred to polyvinylidene difluoride transfer membrane (Amersham Biosciences, Piscataway, NJ, U.S.A.). A DYKDDDK tag horseradish peroxidase-conjugated antibody recognizing the FLAG-tag sequence (Cell Signaling Technology, Beverly, MA, U.S.A.) was used to detect the *HvUGT13248-Flag* protein at a 1:3,000 dilution. The protein was visualized using the SuperSignal West Pico chemiluminescent substrate (Thermo Scientific, Pierce Biotechnology, Rockford, IL, U.S.A.).

Greenhouse screening of transgenic lines against *F. graminearum*.

Plants were sown into Sunshine MVP growth medium (Sun Gro Horticulture, Agawam, MA, U.S.A.) in 6-inch square plastic pots in a greenhouse. Seeds from each transgenic line were planted four seeds per pot. Nontransformed controls (BW, CBO37, Sumai 3, and Wheaton) were also planted four seeds per pot. Plants were fertilized with 1 teaspoon of Osmocote (14-14-14 N-P-K; Scotts Company, Marysville, OH, U.S.A.) fertilizer per pot at the three-leaf stage. ELISA kits (Agdia Inc., Elkhart, IN, U.S.A.) for the *NPTII* antibody were used to identify plants expressing the transgene. At anthesis, one floret of a central spikelet of the main spike was inoculated with 10 µl of *F. graminearum* macroconidial suspension (10⁵ macroconidia per milliliter). Inoculated spikes were covered with transparent plastic bags for 3 days. FHB disease

severity was determined as the percentage of spikelets with disease symptoms on the inoculated spikes at 21 days after inoculation (dai). For statistical analysis, Student's *t* tests were used to compare each transgenic event to the parental nontransformed controls. All analysis was performed with Microsoft Excel 2013 (Microsoft Corp., Redmond, WA, U.S.A.).

Conversion of DON to D3G in planta.

For transgenic event #19 in the BW background and the non-transformed BW control, 10 µl of DON (0.2 µg µl⁻¹) was introduced, at anthesis between the palea and lemma, in each of 25 florets on the main spike of each plant. In this manner, each spike received 50 µg of DON. Three spikes of each genotype were bulk-sampled at 1, 3, 7, 14, and 21 dai. Three biological replications were conducted for each time point for each genotype. The spikes for each replication were ground in liquid nitrogen, and metabolites were extracted in 4× volume of extraction solvent (CH₃CN/H₂O/Hac, 79:20:1). DON and D3G levels were ascertained by LC-MS/MS according to Vendl et al. (2009).

Field screening of transgenic lines against *F. graminearum*.

Field tests were conducted in the summers of 2012, 2013, and 2014 at the University of Minnesota Agricultural Experiment Station (UMore Park) in Rosemount, MN, U.S.A. Seeds for the transgenic events included in the field trials were obtained from ELISA-positive plants in the previous generation. Non-transformed controls (BW, CBO37, Rollag, Wheaton, and Sumai 3) were also planted. Entries were arranged in a randomized complete block design with four replications. All the lines were spray-inoculated twice. The first inoculation was applied at anthesis. The second inoculation was made 3 days after the initial application for each plot. The inoculum was a composite of 30 to 39 *F. graminearum* isolates at a concentration of 100,000 macroconidia per milliliter. The inoculum was applied using a CO₂-powered sprayer fitted with a SS8003 TeeJet spray nozzle with an output of 10 ml per second, at a working pressure of 275 kPa. Mist-irrigation was applied from the first inoculation through disease assessment to facilitate FHB development. FHB incidence and severity were assessed visually 19 to 27 dai on 20 arbitrarily selected heads per plot. FHB incidence was determined by the percentage of spikes with visually symptomatic spikelets of the 20 heads observed. FHB severity was determined as the percent symptomatic spikelets of the total of all spikelets observed. Approximately 60 heads were hand harvested from each plot at maturity, were threshed, and the seeds were cleaned manually. The cleaned grain samples were then used to determine the percentage of VSK (Jones and Mirocha 1999). DON concentrations in the 2012 field trial was conducted by gas chromatography-MS (Fuentes et al. 2005). DON and D3G concentrations of the grain samples in the 2013 and 2014 trials were measured using LC-MS/MS (Vendl et al. 2009).

In the 2013 and 2014 field trials, plant height was measured in the BW and CBO37 transgenic plants and nontransformed controls.

Root growth assay.

Surface-sterilized wheat seeds of the nontransformed BW and CBO37 controls and transgenic events #8, #15, #37, #1381, #1726, #1386, and #1383 expressing *HvUGT13248* were plated on MS growth medium to germinate. One day after germination, seven seedlings of each genotype were transferred to a square petri dish with MS medium containing DON. For BW transgenic plants and the BW nontransformed control, the seedlings were plated on 0, 2, 5, or 10 ppm DON, and, for the CBO37 transgenic plants and CBO37 nontransformed control, the seedlings were plated on 0, 2, and 5 ppm DON. Three replicate plates of each DON concentration were used. The

plates were positioned vertically under white light at room temperature. Pictures were taken of the plates every day from 3 days after germination. Root growth of seedlings was determined by measuring the longest root of each seedling from the photos, using the ImageJ program (Rasband, W.S.; ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, U.S.A.).

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