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UDP-glucosyltransferase HvUGT13248 confers type II resistance to Fusarium graminearum in barley

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(Article begins on next page)

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- 2 Title: HvUGT13248 Confers Type II Resistance to Fusarium graminearum in Barley
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#### 25 Abstract

Fusarium head blight (FHB) of barley (Hordeum vulgare) causes yield losses and accumulation of 26 27 trichothecene mycotoxins (e.g. deoxynivalenol (DON)) in grains. Glucosylation of DON to the 28 nontoxic DON-3-O-glucoside (D3G) is catalyzed by UDP-glucosyltransferases (UGTs), e.g. barley UGT13248. We explored the natural diversity of UGT13248 in 496 barley accessions and 29 30 showed that all accessions tested likely carried functional alleles of UGT13248, as no genotypes 31 tested showed strongly increased seedling sensitivity to DON. From a TILLING population, we 32 identified two mutant alleles (T368I and H369Y) that, based on protein modeling, likely affect UDP-glucose binding of UGT13248. In DON feeding experiments, DON to D3G conversion was 33 strongly reduced in spikes of these mutants compared to controls and plants overexpressing 34 35 UGT13248 showed increased resistance to DON and increased DON to D3G conversion. Moreover, field grown plants carrying the T368I and H369Y mutations inoculated with F. 36 graminearum showed increased FHB disease severity and reduced D3G production. Barley is 37 generally considered to have type II resistance that limits the spread of *F. graminearum* from 38 39 the infected spikelet to adjacent spikelets. Point inoculation experiments with F. graminearum showed increased spread of infection in T368I and H369Y across the spike compared to wild-40 type, while overexpression plants showed decreased spread of FHB symptoms. Confocal 41 microscopy revealed that F. graminearum spread to distant rachis nodes in T368I and H369Y, 42 but was arrested at the rachis node of the inoculated spikelet in wild-type plants. Taken 43 together, UGT13248 confers type II resistance to FHB in barley via conjugation of DON to D3G. 44

45

#### 46 **INTRODUCTION**

Fusarium Head Blight (FHB) is a devastating disease of small-grain cereals and can lead to 47 severe crop losses by reducing crop yield and grain quality (Bai et al., 2018; Chen et al., 2019; 48 49 Johns et al., 2022). It is caused by several species of the fungal genus Fusarium. Fusarium graminearum (teleomorph Gibberella zeae) is considered to be the primary cause of FHB in 50 51 cereal crops globally, especially in wheat (Starkey et al., 2007; Xu and Nicholson, 2009). 52 *Fusarium spp.* produce several groups of mycotoxins, including trichothecenes, zearalenone, 53 beauvericin, enniatins and fumonisins (Ferrigo et al., 2016; McCormick et al., 2011; Santini et al., 2012). Trichothecenes are a large family of sesquiterpenoids defined by their heterocyclic 54 structure, which includes a 9,10-double bond and a 12,13-epoxide (Chen et al., 2019). Type B 55 56 trichothecene mycotoxins, characterized by a keto group at C-8, are produced during Fusariumplant interactions and include deoxynivalenol (DON), nivalenol (NIV), 15-O-acetyl-DON, and 3-57 O-acetyl-DON (McCormick et al., 2011; Varga et al., 2015), of which DON and NIV are among 58 59 the most frequently detected mycotoxins in cereal grains worldwide (Lee and Ryu, 2017). The 60 more recently discovered NX-toxins, such as the prominent NX-3 are structural analogs, but lack the C-8 keto-group (Varga et al., 2015). Trichothecene mycotoxins inhibit protein biosynthesis 61 62 (McLaughlin et al., 1977) and their accumulation is coincident with the switch between biotrophic and necrotrophic growth of F. graminearum, indicating they play an important role 63 in infection (Bushnell et al., 2003). 64

65 The biosynthetic enzymes for trichothecene production are encoded by 15 TRI genes in F. graminearum (Chen et al., 2019). The TRI5 gene encodes trichodiene synthase, which is the 66 first enzyme in the trichothecene biosynthetic pathway, and cyclizes farnesyl pyrophosphate to 67 68 trichodiene (Hohn and Beremand, 1989). The ability of *F. graminearum* to produce 69 trichothecenes is a prerequisite for full pathogenicity and fungal spread in wheat spikes, as tri5 70 mutants can initially infect wheat, but the infection is restricted to the rachis node of the 71 infected spikelet and does not spread throughout the spike (Bai et al., 2001; Jansen et al., 2005; 72 Proctor et al., 1995). Hence, in wheat, tolerance to trichothecenes is required for the ability to 73 limit spread of the infection in the spike (type II resistance), but not resistance to initial infection (type I resistance) (Jansen et al., 2005; Schroeder and Christensen, 1963). Conversion 74

of DON to DON-3-*O*-glucoside (D3G) reduced toxicity on wheat ribosomes (Poppenberger et al.,
2003) and co-segregated with a major FHB resistance QTL (*Fhb1*) in wheat (Lemmens et al.,
2005). UDP-glucosyl transferases (UGTs) catalyze DON to D3G conversion by transferring
glucose from the UDP-glucose substrate to the hydroxyl group of carbon 3 of DON
(Poppenberger et al., 2003). Heterologous expression of the barley *UGT13248* gene in wheat
resulted in increased type II resistance to *F. graminearum*, underscoring the role of DON in
overcoming type II resistance to FHB in wheat (Li et al., 2015).

82 In contrast to wheat, barley is generally considered to have innate type II resistance, and extensive spread of the disease throughout the spike is rare (Boddu et al., 2007; Steffenson, 83 84 2003). Thus, FHB impacts the yield of wheat more than barley. However, because the lemma 85 and palea are retained on barley kernels throughout harvest and processing, barley can accumulate high levels of mycotoxins, which are problematic for human and animal health 86 (Steffenson, 2003). No major FHB resistance locus affecting plant immune signaling or 87 88 biochemistry has been identified in barley to date. FHB resistance in barley is quantitatively 89 inherited, and QTL associated with resistance are also associated with multiple agromorphological traits that influence resistance, including plant height, heading date, spike 90 morphology, including row type and spike density, as well as grain protein content (de la Peña 91 et al., 1999; Huang et al., 2021; Massman et al., 2011; Mesfin et al., 2003; Zhu et al., 1999). 92 93 Interestingly, while wild-type *F. graminearum* grew through the rachis node in the wheat cv. 94 'Nandu' and a tri5 mutant strain was restricted at the rachis node, both of these strains were 95 arrested at the rachis node in the barley cv. 'Chevron' (Jansen et al., 2005). This demonstrates a 96 strong correlation between trichothecene production and FHB disease progression in wheat, 97 while the relationship between FHB disease progression and trichothecene production in barley remains unclear. 98

UDP-glucosyl transferases (UGTs) have been studied and functionally characterized in
 multiple plant species, and some of them have been shown to detoxify DON. In *Arabidopsis thaliana*, a cluster of six UGTs was identified, of which UGT73C5 (DOGT1) and UGT73C4, were
 found to convert DON to D3G (Poppenberger et al., 2003; Schweiger et al., 2010). In barley,
 nine UGT genes were upregulated in response to *F. graminearum* (Boddu et al., 2006), three

104 UGT genes were expressed in response to a wild-type F. graminearum but not a tri5 mutant 105 strain (Boddu et al., 2007) and two UGTs were expressed in DON- but not water-treated barley 106 plants (Gardiner et al., 2010). Four of these barley UGTs, UGT14077, UGT5876, UGT13248 and 107 UGT19290, were expressed in yeast and grown on DON-containing media and only UGT13248 108 was found to convert DON to D3G (Schweiger et al., 2010). Interestingly, the closest homolog to 109 DOGT1 in barley, UGT5876, did not show activity to DON in yeast (Schweiger et al., 2010), 110 suggesting that close protein homology does not necessarily predict substrate specificity in these proteins. Further, UGT14077 was found to glucosylate another *Fusarium* toxin, 111 zearalenone (Michlmayr et al., 2017). Heterologous expression of UGT13248 in Arabidopsis 112 113 thaliana and wheat resulted in DON-resistant seedlings and reduced FHB severity, respectively 114 (Li et al., 2015; Shin et al., 2012). Both transgenic Arabidopsis and wheat rapidly conjugated DON to D3G. UGTs that glucosylated DON were identified in wheat (He et al., 2020; Kirana et 115 al., 2022), Aegilops tauschii (Kirana et al., 2022), rice (Schweiger et al., 2013), sorghum 116 (Schweiger et al., 2013) and Brachypodium distachyon (Schweiger et al., 2013). In B. distachyon, 117 two UGTs Bradi5g03300, the closest Brachypodium homolog to barley UGT13248, and 118 Bradi5g02780 were shown to catalyze DON to D3G conversion in yeast (Schweiger et al., 2013). 119 120 Plants with mutations in Bradi5g03300 showed similar colonization upon point inoculation with 121 F. graminearum as compared to wild-type Brachypodium plants. Moreover, they were more susceptible when spray inoculation was used, suggesting that Bradi5g03300 is involved in type 122 I, but not type II resistance in Brachypodium (Pasquet et al., 2016). Bradi5g03300 123 124 overexpression plants also showed increased root tolerance to DON (Pasquet et al., 2016).

125 Crystal structures for Os79, a rice UGT with high homology to UGT13248, complexed with the nonreactive co-substrate analog UDP-2-deoxy-2-fluoroglucose and trichothecene, 126 which lacks the C3 hydroxyl group to which the glucose molecule is attached, as well as with 127 UDP alone were reported (Wetterhorn et al., 2016). The Os79 structure is similar to other 128 129 known plant UGTs and contain the plant secondary product glycosyltransferase (PSPG) motif, a hallmark of UGT enzymes, which is considered part of the region interacting with the UDP-sugar 130 131 co-substrate (Gachon et al., 2005; Vogt and Jones, 2000; Wetterhorn et al., 2016). The active 132 site of Os79 is located in a cleft between the C-terminal donor-binding and the N-terminal

133 acceptor domains (Wetterhorn et al., 2016). Using structural alignments, it was shown that 134 Os79 likely utilizes a catalytic mechanism similar to those of other plant UGTs, where His 27 135 activates the trichothecene O3 hydroxyl for nucleophilic attack at C1 of the UDP-glucose donor 136 molecule (Wetterhorn et al., 2016). Kinetic analysis of Os79 mutants identified Thr 291 as required to position the UDP moiety during the nucleophilic attack or for catalysis as a catalytic 137 acid (Wetterhorn et al., 2016). Os79 conjugates multiple trichothecene substrates such as DON, 138 139 nivalenol, isotrichodermol, and HT-2 toxin, but not T-2 toxin (Wetterhorn et al., 2016). Interestingly, three mutations in Os79 yielded a H122A/L123A/Q202L Os79 triple mutant that 140 had an increased active site volume and showed activity on T-2 toxin (Wetterhorn et al., 2017). 141 142 UGT13248 was able to conjugate nivalenol, whereas DOGT1 was not (Li et al., 2017).

143 DON is a virulence factor in wheat and detoxification of DON is clearly linked to increased type II resistance. However, it is unclear if this is true in other grasses, including B. 144 distachyon and barley. We decided to study UGT13248 directly in barley to understand if 145 detoxification of DON by conversion to D3G affects FHB disease progression in this crop. We 146 147 first investigated natural variation in UGT13248 to identify UGT13248 variants with altered function. In 496 barley accessions tested, no UGT13248 alleles that abolished function were 148 identified, suggesting that UGT13248 function is highly conserved throughout the species. We 149 150 next identified mutations with reduced UGT13248 function and plants overexpressing 151 UGT13248 to study the role of UGT13248 in barley-Fusarium interactions in detail. We found 152 that UGT13248 confers type II resistance to *F. graminearum* in barley.

153

#### 154 **RESULTS**

#### 155 Many barley accessions contain functional UGT13248 alleles

156 Our previous research showed that barley UGT13248 converts DON to D3G in wheat,

- 157 Arabidopsis and yeast (Li et al., 2015; Schweiger et al., 2010; Shin et al., 2012). Overexpression
- 158 of UGT13248 in wheat resulted in rapid DON to D3G conversion and reduced FHB prevalence (Li
- et al., 2015). Here, we aimed to understand the function of UGT13248 directly in barley.
- 160 Multiple groups have identified QTLs in barley that affect DON accumulation and FHB severity

(Huang et al., 2018; Massman et al., 2011); however, the QTLs found generally affected agro-161 162 morphological traits including plant height, heading date, or spike morphology (Massman et al., 163 2011; Mesfin et al., 2003; Zhu et al., 1999). UGT13248 has not been identified as a contributor 164 to FHB resistance in any of these QTL studies (Huang et al., 2018; Massman et al., 2011). We decided to screen a natural population of barley accessions for mutations in UGT13248 that 165 might affect UGT13248 function. First, we compared the UGT13248 protein sequences of eight 166 167 susceptible and 18 resistant barley accessions (Supplemental Table 1; (Huang et al., 2013). We considered the UGT13248 sequence of cv. 'Morex', used for the first barley whole genome 168 sequencing project (Mayer et al., 2012), the wild-type allele, as UGT13248 was identified 169 170 initially in this cultivar (GU170355; (Schweiger et al., 2010). In these 26 accessions, two nonsynonymous mutations were found. A104V was found in seven accessions and G213R in two 171 accessions (Supplemental Figure 1A and Supplemental Table 1). Of these, only G213R was 172 173 exclusively found in two accessions that are susceptible to FHB, PI383933, and ICB111809 (Huang et al., 2013). We grew representative accessions that carried UGT13248 alleles 174 representing each mutation, and wild-type Morex plants on mock and DON-containing media 175 and measured seedling root length for six days. While seedling root length on DON-containing 176 177 media was reduced in the cv. 'Fredrickson' that carried the A104V mutations, other accessions 178 carrying the same mutation did not show significantly reduced root growth (Supplemental Figure 1 B-D). None of the accessions containing mutations in *UGT13248* showed severely 179 reduced root growth on DON-containing media (Supplemental Figure 1 B-D), suggesting that 180 none of the mutations had a drastic effect on DON-induced root growth inhibition. 181

We next analyzed the *UGT13248* sequences of 19 additional barley accessions, sequenced as part of a barley pan-genome analysis (Jayakodi et al., 2020). We identified A104V in six additional accessions and G213R in seven additional accessions (Supplemental Figure 1A and Supplemental Table 1). We also identified an additional mutation in the accession FT 11/ B1K-04-12, corresponding to R47H (Supplemental Figure 1A). No difference in seedling root growth was observed between mock and DON-containing media for FT11 (Supplemental Figure 18). 189 We next utilized protein modeling as a predictive tool to develop hypotheses if these 190 mutations potentially affect UGT13248 function. SWISS MODEL blast identified the closest 191 homolog to UGT13248 at 73 % amino acid sequence identity as rice Os79, for which a crystal 192 structure has been resolved (Wetterhorn et al., 2016). We aligned the sequence used for the 193 Os79 crystal structure (PDB ID: 5TMD), the full-length Os79 protein sequence, and the 194 UGT13248 protein sequence (Supplemental Figure 2A). We used the Os79 three-dimensional 195 structure with trichothecene and the nonreactive co-substrate analog UDP-2-deoxy-2-196 fluoroglucose modeled within the active site cavity as a template for barley UGT13248 protein 197 models. The resulting homology models of UGT13248 exhibited a similar Rossman-fold type 198 three-dimensional structure as Os79. In Os79, the proposed catalytic residues for initiating the 199 glycosylation of trichothecene via deprotonation of the hydroxyl group are H27 and D120, while 200 T291 is hypothesized to be involved in coordinating the UDP during the subsequent nucleophilic attack. Based on sequence alignments, the corresponding UGT13248 residues that could be 201 hypothesized to act as the catalytic dyad are H38 and D130, and T299 could serve as the UDP-202 203 coordinating group (Supplemental Figure 2A). The homology models indicated that these three residues would likely be structurally positioned within an active site pocket similar to that of 204 205 Os79 and, therefore may be important for the correct function of UGT13248 (Supplemental 206 Figure 2B). The structural positions of mutated residues in models of R47H, A104V, and G213R, indicated that these amino acids are not within close proximity with the UDP-glucose and 207 trichothecene molecules (Supplemental Figure 2C-E). It was therefore considered less likely that 208 these mutations would influence UGT13248 function. 209

210 To explore the genetic diversity of UGT13248 in a larger natural population, we utilized previously-generated exome capture sequencing data (Chen et al., 2022; Hemshrot et al., 2019; 211 Kono et al., 2019; Lei et al., 2019; Mascher et al., 2013; Nice et al., 2016; Russell et al., 2016) to 212 screen for mutations in UGT13248. The data set included 83 elite lines, 188 wild barley 213 accessions and 187 landraces. The exome capture sequencing data covered two regions of the 214 Morex UGT13248 gene. One region covered a portion of exon 1 and intron 1, and the other 215 216 region covered the majority of exon 2, intron 2, and exon 3 (Supplemental Figure 3A). The 217 second region contains the PSPG box (Gachon et al., 2005; Vogt and Jones, 2000), thought to be 218 important for UDP-glucose binding, as well as the predicted residue for UDP positioning T299 219 that had been identified during homology modeling (Supplemental Figure 2A). From this 220 analysis, nine non-synonymous mutations were identified in 64 of these 458 accessions. These 221 nine non-synonymous mutations were G213R, M281I, D282N, F385S, S403I, L419V, Y440L, 222 S461P and F468L (Supplemental Figure 3A). G213R, which we identified in nine of the 45 barley 223 accessions with full-length genomic DNA sequence data (Supplemental Table 1), was found in 224 an additional 38 landraces and 3 elite cultivars using the exome capture sequencing approach (Supplemental Table 2). The other eight non-synonymous mutations were identified in seven 225 landraces and 16 wild barley accessions (Supplemental Table 2). Analysis of the protein 226 227 homology models of these mutants indicated that the mutated residues M281I, D282N, F385S, 228 S403I, L419V, Y440L, S461P, and F468L were not structurally positioned within the catalytic site, leading us to hypothesize that none of these mutations would directly influence UGT13248 229 catalytic function (Supplemental Figure 3B-I). To confirm, we screened 14 accessions, 230 231 representing these other eight non-synonymous UGT13248 mutations, and measured seedling root growth on mock and DON-containing media. Seedling root length on DON-containing 232 media was slightly, but significantly reduced in FT222, which carries the M281I mutation, FT735 233 234 that carries the F468L mutation, as well as two of six accessions that carry the D282N mutation, 235 Hor2867, and PI57099 (Supplemental Figure 4 A, B, D and E). None of the accessions tested however exhibited a complete inhibition of root growth upon DON exposure (Supplemental 236 Figure 4), suggesting that the mutations M281I, D282N, F385S, S403I, L419V, Y440L, S461P, and 237 238 F468L did not have a deleterious effect on UGT13248-dependent DON to D3G conversion. Overall, none of the 11 mutations, identified in the 496 accessions analyzed, had a strong 239 adverse effect on UGT13248 activity and all these lines likely contained functional UGT13248 240 alleles. Taken together, we conclude that mutations in UGT13248 are rare and likely do not 241 242 disrupt UGT 13248 protein function.

243

244 **Mutations in UGT13248 or constitutive expression of UGT13248 affect root sensitivity to DON** 245 To further study the role of UGT13248 in barley, we decided to utilize plants that constitutively 246 express UGT13248. We transformed *UGT13248* under the control of the constitutive *Zea mays*  247 *Ubiquitin* (*Ubi-1*) promotor into the barley cultivar 'Golden Promise' (Supplemental Figure 5A). We identified three independent insertion lines in Golden Promise (Supplemental Figure 5B). As 248 249 Golden Promise does not reliably flower under our growth conditions, we crossed the 250 transgenic lines with the cultivar 'Rasmusson'. We backcrossed the plants once with Rasmusson 251 and then identified sets of sister lines that contained and expressed the UGT13248 transgene 252 transcript and the UGT13248 protein (UGT+) or did not contain either (UGT-) from two 253 independent transgenic events (#39003 and #39009) (Supplemental Figure 5C and E). For both 254 sets of sister lines the accession containing the transgene and expressing UGT13248 (UGT+) did 255 not show significantly reduced seedling root growth two to six days after DON treatment, while 256 the non-transgenic UGT-lines, as well as Golden Promise and Rasmusson wild-type seedlings 257 did (Figure 1A and B). We conclude that overexpression of UGT13248 increases root resistance to DON application. 258

259 We also screened a TILLING population in the barley cultivar Morex (TILLMore; (Talame et al., 2008a) for UGT13248 mutations. We identified three lines that contained non-260 261 synonymous mutations T368I, H369Y, and S403N. Two of the three lines, T368I and H369Y, exhibited strongly reduced seedling root growth two to six days after transfer to DON-262 263 containing media when compared to wild-type Morex plants (Figure 1C and D). S403N plants were indistinguishable from Morex plants (Figure 1C). Additionally, we crossed the H369Y and 264 T368I plants to Morex and analyzed the resulting F<sub>2</sub> population. Upon exposure to DON the 265 roots of homozygous T368I and H369Y mutants were significantly shorter than roots of 266 267 homozygous wild-type and heterozygous plants (Supplemental Figure 6A and B). We conclude 268 that plants carrying the T368I and H369Y mutations of UGT13248, but not the S403N mutation, 269 have reduced UGT13248 function.

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# Protein models suggest that UGT13248 T368I and H369Y variants might have altered substrate binding

To further understand the effect of the T368I and H369Y mutations on UGT13248 function, we
created protein models for UGT13248 carrying the T368I, H369Y, and S403N mutations and

275 compared these to the wild-type UGT13248 protein model (Figure 2). According to the protein 276 models that were generated, amino acids T368 and H369 are located within 4 Å of the UDP-277 glucose molecule. Thus, we hypothesized that these two residues may contribute to the hydrogen bonding network for the stabilization of UDP at the active site (Figure 2). In the Os79 278 279 crystal structure, residue T291 was proposed to be involved in either the positioning of the substrate, or in protonation (Wetterhorn et al., 2016). In the UGT13248 protein homology 280 281 model, T368 is positioned within hydrogen bonding distance of H369, which in turn is positioned within hydrogen bonding distance of UDP-glucose. Therefore, T368 and H369 may 282 be required for positioning of substrate within the active site. On the other hand, in the 283 284 UGT13248 protein homology model, S403 is distant (>4 Å) from the active site and is therefore not expected to be involved in substrate binding. This may explain why the mutant S403N roots 285 did not show increased susceptibility to DON. 286

Interestingly, we noted in the protein model that when the polar residue threonine is replaced by a hydrophobic isoleucine (T368I), the hydrogen bond between a hydroxyl side chain and the nitrogen atom of H369 is disrupted, which could potentially indicate an impact on the hydrogen bonding network surrounding the substrate. Furthermore, when the charged residue histidine is replaced by hydrophobic tyrosine (H369Y) in the protein model, the negatively charged hydroxyl group of the tyrosine side chain has the potential to cause steric hindrance of UDP, possibly inhibiting correct substrate binding within the active site (Figure 2).

Taken together, these differences in the protein models suggest that the T368I and H369Y mutations could affect UGT134248 function by influencing the binding of the UDPglucose substrate, potentially impacting the enzyme function, which may explain the increased sensitivity to DON demonstrated in plants carrying these mutations.

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#### 299 UGT13248 is required for DON to D3G conversion

To test the effect of UGT13248 overexpression on DON to D3G conversion, we inoculated spikes of #39003\_UGT- and #39003\_UGT+ sister lines with DON at anthesis. We measured DON and D3G levels 0 h, 4 h, 24 h, 48 h, and 96 h after inoculation. UGT- plants that do not contain 303 the transgene, showed some increase in D3G / DON over time, while the D3G / DON ratio was 304 significantly increased in UGT+ plants at 24 h, 48 h, and 96 h (Figure 3A). To better understand 305 the dynamics of UGT13248 expression in these genotypes, we used gRT-PCR to compare 306 expression of the endogenous UGT13248 and the UGT13248-FLAG transgene in mock- and 307 DON-treated UGT- and UGT+ plants (Supplemental Figure 5D). UGT+ and UGT- plants showed similar levels of endogenous UGT13248 in mock treated samples at 0 h, 4 h, and 24 h after 308 309 inoculation. In UGT- plants a strong increase in endogenous UGT13248 was detected at 4 h and 24 h after DON inoculation. In UGT+ plants an increase of endogenous UGT13248 expression 310 was detected at 4 h, but not 24 h after DON application. In addition to the endogenous 311 312 UGT13248, UGT+ plants expressed the UGT13248-FLAG transgene at a constitutive and 313 elevated level compared to endogenous UGT13248. The total amount of UGT13248 expression in UGT+ plants is higher compared to UGT- plants at 0 h, 4 h, and 24 h after mock treatment 314 and at 0 h and 4 h after DON application. The combined level of UGT13248-FLAG and 315 316 endogenous UGT13248 expression in UGT+ plants is similar to the endogenous UGT13248 expression in UGT- plants at 24 h after DON treatment. This suggests that UGT13248 expression 317 is upregulated upon DON application. It is possible that the level of UGT13248 expression is 318 319 regulated by a feedback mechanism that prohibits continued UGT13248 expression in response 320 to DON after a certain UGT13248 expression level is reached or alternatively, DON to D3G conjugation might reduce available DON concentrations and thus reduce DON-induced 321 322 UGT13248 gene expression. Taken together, the data show that UGT13248 converts DON to 323 D3G in barley and that earlier and higher levels of UGT13248 lead to increased conversion of DON to D3G. 324

To understand the effect of altered UDP-glucose binding on UGT13248 function in more detail, T368I, H369Y, S403N, and Morex spikes were inoculated with DON at anthesis. DON and D3G were measured at 0 h, 12 h, 24 h, 48 h, and 96 h after inoculation. T368I and H369Y plants showed significantly reduced D3G / DON accumulation at 24 h, 48 h, and 96 h, compared to Morex and S403N plants (Figure 3B). This shows that the T368I and H369Y mutations inhibit UGT13248-dependent DON to D3G conversion.

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#### 332 UGT13248 mutants have increased susceptibility to FHB in the field

We performed field evaluations to investigate the effect of the reduced UGT13248 activity on 333 334 FHB severity and DON and D3G accumulation upon plant infection with *F. graminearum*. Field 335 evaluations of Morex, T368I, H369Y, and S403N plants were performed in two years, 2019 and 336 2020, and in two different field locations in Minnesota, Saint Paul and Crookston. No 337 differences in plant height were observed between Morex and any of the mutants in three 338 environments (Supplemental Figure 7A-C). In Saint Paul, plants were spray-inoculated at 339 anthesis and in Crookston grain spawn inoculation was used. In all four environments T368I 340 plants showed significantly increased FHB severity compared to wild-type Morex, while S403N plants did not. In three out of four environments (Saint Paul, 2020, Crookston, 2019 and 341 Crookston, 2020) H369Y plants showed significantly increased FHB severity compared to wild-342 type Morex (Figure 4A and B). FHB disease development is influenced by many factors including 343 temperature and humidity thus it is not unusual to see variation of FHB severity in different 344 environments (Steffenson, 2003). We conclude that H369Y and T368I plants, that have strongly 345 346 reduced UGT13248 activity, are more susceptible to *F. graminearum*.

DON and D3G accumulation were measured in plants from the Saint Paul trial in 2019 and 2020 (Supplemental Figure 7D-G). In both years, the ratio of D3G to DON was significantly reduced in T368I and H369Y plants compared to wild-type Morex and S403N plants from 14 days after inoculation to harvest (30-32 days after inoculation) (Figure 4C and D). Thus, even in the environment that we did not detect any increased FHB severity for H369Y (Saint Paul 2019), we still detected a robust reduction in the conversion of DON to D3G in H369Y plants under field conditions.

354

#### 355 **Point inoculation experiments suggest a role of UGT13248 in type II resistance in barley**

356 It has previously been shown that *F. graminearum* produces DON during the infection process

357 (Evans et al., 2000) and that trichothecenes are virulence factors in wheat (Proctor et al., 1995).

To gain a more detailed understanding of the effect of UGT13248 on FHB disease progression in

barley, we used point inoculation experiments on UGT13248 mutants and overexpression

360 plants. We inoculated the two central spikelets in the middle of each spike on either side of the spike with F. graminearum strain PH-1 and monitored FHB severity over time. In both sets of 361 362 UGT13248 overexpression plants (#39003 and #39009), the UGT+ individuals, carrying the 363 UGT13248 transgene, showed significantly increased resistance to *F. graminearum* at 7 and 11 days after inoculation compared to the UGT- sister lines (Figure 5B). The UGT+ (39009) plants 364 365 also showed significantly decreased susceptibility at 14 and 21 days after infection compared to 366 their UGT- (39009) sister line, while no significant difference could be detected between the second set of sister lines (39003) (Figure 5A and B). In the case of the 39003 sister lines, the 367 UGT+ plants were shorter and showed increased spike density compared to the UGT-368 369 individuals (Supplemental Figure 8), which likely increased disease progression at the later time 370 points in the UGT+ plants. We conclude that UGT13248 overexpression leads to increased type 371 Il resistance to F. graminearum at least early after infection with F. graminearum.

372 Next, point inoculation experiments were performed for Morex, T368I, H369Y, and 373 S403N plants. While FHB symptoms were restricted to one to two rows of spikelets basipetal 374 and acropetal each from the inoculated spikelets in Morex and S403N, symptoms spread much further in T368I and H369Y plants (Figure 5C). T368I and H369Y plants, which have strongly 375 376 reduced UGT13248 activity showed significantly increased FHB severity at 7, 11, and 14 days after inoculation when compared to wild-type Morex and S403N plants (Figure 5D). We 377 conclude that DON to D3G conversion by UGT13248 is a major contributor to type II resistance 378 379 in barley.

380

### 381 Differences in susceptibility to *F. graminearum* in the field are not primarily dependent on 382 differences in type II resistance

We utilized a set of barley genotypes to determine if point inoculation can reproduce differences in FHB severity previously described under field conditions. We inoculated the resistant cultivars Chevron, Quest, Stellar, and Frederickson and the susceptible accessions Morex, PI383933, ICB111809, and Stander, together with the H369Y plants. Only the highly susceptible accession PI383933 (Huang et al., 2013) and H369Y plants showed very high FHB 388 severity, while all other genotypes showed comparable FHB severity (Supplemental Figure 9A). 389 We also plotted the average number of diseased spikelets in each genotype (Supplemental 390 Figure 9B) as this may be a truer representation of fungal spread as different genotypes had 391 different total numbers of spikelets or rachis nodes, and the tested genotypes were comprised 392 of 2-row and 6-row accessions. The 2-row accessions Fredrickson and ICB111809 showed the 393 lowest number of diseased spikelets, the 6-row accessions Chevron, Morex, Stander and Stellar 394 showed slightly higher numbers of diseased spikelets, the 6-row accession PI383933 showed a 395 higher number of diseased spikelets and the 6-row accession Quest showed an intermediate number of diseased spikelets compared to PI383933 and Chevron, Morex, Stander and Stellar. 396 397 However, the number of diseased spikelets was significantly higher in H369Y plants compared 398 to all other genotypes tested (Supplemental Figure 9B).

PI383933 plants are very short and have short, dense spikes ((Huang et al., 2018), 399 Supplemental Figures 8 C-E). While PI383933 plants contain the G213R mutations of UGT13248, 400 401 PI383933 seedlings did not show high sensitivity to DON (Supplemental Figure 1C), suggesting 402 that they carry a functional UGT13248 allele. Additionally, in a mapping population of a cross 403 between Rasmusson and PI383933 (Huang et al., 2018), taller plants were generally more 404 resistant to FHB and shorter plants were generally more susceptible to FHB. This was 405 independent of the Rasmusson or PI383933 UGT13248 allele they carried, but dependent on 406 plant height (Supplemental Figure 10 B). We also observed that the spikelets on the tip of the PI383933 spikes remained green in individuals with less than 100% FHB severity, while the 407 408 terminal florets in H369Y plants were bleached (Supplemental Figure 10 A). Thus, susceptibility 409 of PI383933 to F. graminearum is likely due to plant morphology rather than reduced type II 410 resistance.

Taken together, this suggests that FHB susceptibility in the field is not likely predominantly dependent on differences in type II resistance, but rather type I resistance, rowtype, and various agro-morphological traits that do not affect FHB development upon point inoculation under controlled growth conditions.

415

## 416 *Fusarium graminearum* spreads through the rachis of T368I and H369Y but is restricted to the 417 inoculated rachis node in wild-type barley plants

418 Previously, Jansen et al. (Jansen et al., 2005) studied the spatiotemporal dynamics of F. 419 graminearum infection in wheat and barley using a GFP-labeled wild-type F. graminearum 420 strain as well as a strain lacking trichothecene production. The authors found that in barley 421 both the wild-type and the trichothecene knockout strain were inhibited at the rachis node of 422 the infected spikelet. We used the H369Y and Morex plants to study if UGT13248 was required 423 for blocking *F. graminearum* invasion of the rachis. We inoculated the two central spikelets on either side and in the middle of each spike with *F. graminearum* strain PH-1 and 21 days later 424 collected rachis and florets separately from five equal sections of each Morex and H369Y spikes 425 426 (Figure 6A-C). Each spike was separated into five equal portions based on the total number of rachis nodes. Section 1 was on the base of the spike and section 5 at the tip. Section 3 427 contained the inoculated central spikelets, the lateral spikelets adjacent to the inoculated 428 429 spikelets, and all central and lateral spikelets of the rachis nodes directly acro- and basipetal of 430 the inoculated spikelets. For example, if the spike contained 60 spikelets, there were 12 spikelets and four rachis nodes in each section. For each section, ergosterol and DON 431 432 accumulation was measured separately for floret and rachis tissue. Ergosterol is a major sterol 433 found in fungal cell membranes and was used as a proxy for *F. graminearum* quantity. Ergosterol accumulation was significantly increased in section 4, located acropetal to the 434 435 inoculated spikelets, of H369Y plants compared to wild-type Morex. H369Y plants also 436 contained significantly more ergosterol in the rachis sections two and three compared to Morex 437 (Figure 6A and C). DON levels were significantly higher in H369Y florets in sections two, three, and four as well as H369Y rachis in sections three and four (Figure 6B and C). We conclude that 438 H369Y plants contain more DON and F. graminearum than wild-type Morex, specifically in the 439 rachis near the inoculation site. 440

Next, we decided to study symptom development in H369Y and Morex plants upon
inoculation with a *F. graminearum* reporter strain that constitutively expressed dsRed
(*pgpdA::dsRed*) (Ilgen et al., 2009). For these experiments, we only inoculated one central floret
in the middle of the spike and reduced the *F. graminearum* inoculum by half to reduce the

445 chance of mycelium development on the outside of the spike. Spread of FHB symptoms and 446 overall FHB severity 14 days after inoculation were strongly increased in H369Y spikes 447 compared to wild-type Morex (Supplemental Figure 11A-D). Increased FHB severity was 448 observed after point inoculation of homozygous H369Y plants 7 and 21 days after inoculation, 449 but not in corresponding sister lines (Supplemental Figure 11E). In T368I and H369Y mutant 450 plants browning of the rachis was observed over the entire length of the rachis, but not in the 451 corresponding wild-type sister lines. We used confocal laser scanning microscopy to visualize fungal mycelia at base of the spike and observed structures that resembled fungal hyphae in 452 higher magnification images of both T368I and H369Y mutants plants, while no fungal signal 453 454 was observed in the corresponding wild-type tissues (Figure 7A). Next confocal laser scanning microscopy was used to visualize the infection process in H369Y and Morex plants in more 455 456 detail. While F. graminearum could be detected at the rachis nodes directly at the inoculated spikelets in both H369Y and Morex, F. graminearum was only detected at the fourth rachis 457 nodes acro- and basipetal of the inoculated spikelets in H369Y, but not Morex (Figure 7B and 458 459 Supplemental Figure 12). Additionally, F. graminearum was detected throughout the rachis 460 node and all rachis tissue at the inoculated rachis node in H369Y. In contrast F. graminearum 461 was detected only locally at the rachis node of the inoculated spikelet in wild-type rachis tissue 462 and not in more distant parts of the rachis (Figure 7B and Supplemental Figure 12). This shows that F. graminearum does infect the rachis of H369Y plants, but is arrested at the inoculated 463 rachis node in wild-type plants. Examination of the susceptible PI383933 plants showed a 464 similar pattern to wild-type plants (Supplemental Figure 10C). We conclude, UGT13248-465 dependent detoxification of DON is required for type II resistance in barley. 466

467

#### 468 DISCUSSION AND CONCLUSIONS

469

#### 470 UGT13248 function in barley is highly conserved

471 Here, we screened 45 barley accessions for natural variation in full-length UGT13248

472 (Supplemental Table 1) and 458 accessions for natural variation in two regions of UGT13248

473 covered by exome capture sequencing (Supplemental Table 2). The accessions tested include 474 modern cultivars, landraces, and wild barleys. None of the 11 non-synonymous mutations 475 identified, resulted in disruption of DON resistance in barley seedlings carrying these mutations 476 (Supplemental Figures 1 and 4). Only one of these mutations, F385S, was located within the 477 PSPG motif, thought to be required for UGT-UDP-sugar interaction (Supplemental Figure 3A). 478 Further, protein modeling suggested that none of the mutations was likely to disrupt UGT13248 479 protein function (Supplemental Figures 2 and 3). Interestingly, the only UGT13248 mutations with reduced UGT13248 function were H369Y and T368I, found in a sodium azide-mutagenized 480 TILLING population of barley cultivar Morex (Figure 1). We conclude that UGT13248 gene 481 482 function is highly conserved in barley.

483

#### 484 UGT13248 converts DON to D3G in barley

485 It has previously been shown that UGT13248 expression in yeast and wheat resulted in the 486 conversion of DON to D3G (Li et al., 2015; Schweiger et al., 2010). Here, we observed that DON to D3G conversion in barley spikes that carried T368I and H369Y mutations was significantly 487 488 reduced compared to wild-type barley (Figure 3B). We also demonstrated that constitutive 489 expression of UGT13248 under the control of the Zea mays Ubiquitin (Ubi1) promoter resulted in strongly increased DON to D3G conversion (Figure 3A). This further shows that UGT13248 490 491 catalyzes DON to D3G conversion in planta. While UGT13248 expression was induced by DON 492 treatment in both barley spikes with wild-type and constitutive UGT13248 expression, low-level 493 constitutive UGT13248 expression was observed even in plants that did not carry the Ubi-494 1::UGT13248 transgene (Supplemental Figure 5D). This suggests that UGT13248 expression is 495 upregulated upon DON application and that the level of UGT13248 expression might be 496 regulated by a feedback mechanism that prohibits continued UGT13248 expression in response to DON after a certain UGT13248 expression level is reached. 497

UGT13248 has been shown to catalyze NIV to NIV3G conversion in wheat (Li et al.,
2017). Interestingly, *Arabidopsis* DOGT1 (UGT73C5) showed activity on DON and the growth
hormone brassinolide, but not NIV (Poppenberger et al., 2005). Another Arabidopsis UGT,

501 UGT73C6, converted zearalenone to ZON-4-O-Glc in yeast (Poppenberger et al., 2006). The 502 closest homolog to DOGT1 in barley UGT5876, did not show activity to DON in yeast (Schweiger 503 et al., 2010). This shows that UGTs are promiscuous and might have multiple substrates. It is 504 possible that UGT13248 can glucosylate other trichothecenes in barley, as well as potentially 505 unrelated compounds including growth regulators and mycotoxins produced by other fungal 506 pathogens. However, constitutive expression of UGT13248 did not lead to any detectable 507 pleiotropic effects. Hence, it is unlikely that UGT13248 drastically affects plant growth regulation, as opposed to DOGT1 in Arabidopsis where overexpression plants showed a dwarf 508 phenotype (Poppenberger et al., 2005). 509

510

#### 511 UGT13248 is a major resistance factor in barley to Fusarium graminearum

Barley carrying mutations in UGT13248 that caused reduced UGT13248 function, H369Y and
T368I, are highly susceptible to *F. graminearum*, both in field studies and in point inoculation
experiments (Figures 4 and 5). The difference in FHB severity is clearest with point inoculation
experiments (Figure 5). We conclude that UGT13248 is required for type II resistance to *F. graminearum* in barley. Our experiments cannot distinguish between type I and type II
resistance in the field and we cannot rule out an additional effect of UGT13248 on type I
resistance in field studies. The conserved type II resistance observed for this species

519 (Steffenson, 2003) might be due to conserved UGT13248 function.

520 Previously, the *B. distachyon* UGT Bradi5g03300, the closest *Brachypodium* homolog to 521 barley UGT13248, was suggested to not contribute to type II resistance in that species (Pasquet 522 et al., 2016). Mutations in Bradi5g03300 only caused increased colonization with F. 523 graminearum when spray inoculation was used. In contrast, point inoculation experiments did 524 not show any difference in FHB severity between plants with mutations in Bradi5g03300 525 compared to wild-type plants (Pasquet et al., 2016). The mutations in Bradi5g03300 are located within the PSPG box similar to the H369Y and T368I mutations in barley UGT13248 studied here 526 527 (Supplemental Figure 13). The observed differences may be due to differences in flower 528 morphology in *B. distachyon* compared to barley. It is also possible that the study of

529 Bradi5g03300 was complicated by the presence of Bradi5g02780, another UGT that showed 530 DON resistance in yeast (Schweiger et al., 2013), and that may be functionally redundant with 531 Bradi5g03300. The differences observed in the function of UGTs in this study compared to *B*. 532 *distachyon* show that studies in model organisms may not always be predictive of the 533 mechanism observed in related crop species.

In wheat, a few major contributors to FHB resistance, including *Fhb1* and *Fhb7*, have been identified (Bai et al., 1999; Wang et al., 2020). However, in barley, FHB resistance has previously been found to be multifactorial and mainly dependent on agro-morphological traits (Massman et al., 2011). Hence, our identification of a major contributor to type II resistance in barley is notable. Interestingly, previous barley mapping studies have not identified a QTL associated with FHB resistance and coincident with *UGT13248*, which is likely due to functional conservation of UGT13248.

541 While barley UGT13248 was found to play a critical role in plant immunity to F. graminearum, it is curious why UGT13248 appears functionally conserved globally in barley, as 542 not all growth regions are likely substantially affected by FHB outbreaks. However, various plant 543 pathogenic fungi of the genera Fusarium, Microcyclospora, Myrothecium, Peltaster, Spicellum, 544 Stachybotrys, Trichoderma, and Trichothecium produce trichothecene mycotoxins (Proctor et 545 al., 2018). Up to 87% of tested food and feed samples from the Middle East and Africa 546 547 contained type B trichothecenes (Rodrigues et al., 2011). It is possible that UGT13248 can 548 detoxify trichothecene mycotoxins from various fungal pathogens and might hence contribute to disease resistance in many different environments. It can also not be ruled out that 549 UGT13248 glucosylates other unknown compounds that are unrelated to trichothecenes. 550

Interestingly, in *Aegilops tauschii* nine of 147 accessions tested showed increased FHB susceptibility and increased FHB spread (Kirana et al., 2022). These nine accessions all carried a nonsense mutation just prior to the PSPG box in the UGT AET5Gv20385300 (Supplemental Figure 13), an ortholog of barley UGT13248 (Kirana et al., 2022). *A. tauschii* is the D subgenome donor for bread wheat and the sequenced cultivar Chinese Spring did also contain a missense mutation in the wheat ortholog of UGT13248 in the D genome (Kirana et al., 2022). This again raises the question why UGT13248 is functionally conserved globally in barley but not in theserelated species.

559

#### 560 **DON detoxification is required for type II resistance to FHB in barley**

Trichothecene production is known to be required for *Fusarium graminearum* spread in wheat 561 562 spikes (Jansen et al., 2005; Schroeder and Christensen, 1963). In the barley cultivar 563 'Chevron', however, both wild-type and tri5 mutant strains of F. graminearum were arrested at the rachis node of the inoculated spikelet (Jansen et al., 2005). Utilizing functional mutations in 564 565 UGT13248, H369Y and T368I, we showed that functional UGT13248 is required for the arrest of 566 F. graminearum at the inoculated rachis node in barley (Figure 7 and Supplemental Figures 11 567 and 12). Hence, the functional conservation of UGT13248 across barley may explain the conserved type II resistance observed for this species (Steffenson, 2003). 568

569

#### 570 MATERIALS AND METHODS

#### 571 Plant Material and growth conditions

572 Plants were grown at 20°C, 16 h daylight, and 8 h dark at 18°C in BM2 soil (Berger) with 3-4

573 seedlings per five-inch square pot. After seedling emergence, plants were fertilized with

574 Osmocote plus fertilizer (Scotts). The plant genotypes used in this study are summarized in

575 Table 1.

576

#### 577 Transgenic barley plants

The *UGT13248* gene was previously cloned with a *C*-terminal FLAG tag into pENTR TM/ D TOPO (Shin et al., 2012) and transferred into binary vector pIPKb002 (Himmelbach et al., 2007) using Gateway cloning technology (Invitrogen). Immature barley cultivar Golden Promise embryos were transformed using the *Agrobacterium tumefaciens* strain AGL1 and transformants were selected on 50 mg / L hygromycin containing media (Hensel et al., 2008). T1 plants were screened for the presence of the transgene using UGT13248 fwd and FLAG rev primers (Supplemental Table 3). For DNA gel blotting genomic DNA (10 µg) was digested with Xbal, separated on a 1 % agarose gel, and transferred onto Hybond N+ membranes (Amersham Biosciences). The *hpt* gene probe was derived from a PCR-amplified product (Supplemental Table 3). The probe was labeled with  $\alpha$ -<sup>32</sup>P CTP using the Prime-a-Gene labeling system (Promega), following the manufacturer's instructions. The radiolabeled *hpt* probe was used for the hybridization, and results were visualized using autoradiography.

590 One transgenic plant each from two independent transgenic events was crossed to 591 barley cultivar Rasmusson and F<sub>1</sub> plants were backcrossed to Rasmusson. In the F<sub>3</sub> generation, 592 individuals containing the UGT13248-FLAG (UGT+) and individuals not containing the transgene 593 (UGT-) were identified. All experiments were conducted using grains that descended from a 594 single individual plant and were used in the BC<sub>1</sub>F<sub>6</sub> generation.

595 For Western Blot analysis one individual seedling for each genotype was harvested and 596 tissue was ground in liquid nitrogen. Plant tissue was mixed with equal volumes of 2 x Laemmli 597 buffer, heated at 95°C for 5 minutes and supernatant was loaded onto a 10% polyacrylamide 598 gel (Biorad, USA, 1610182). Stain-free technology was used to visualize equal loading of the gel. 599 The proteins were transferred onto a PVDF membrane and Western Blot analysis was 600 performed according to the manufacturer's instructions using an anti-FLAG HRP antibody 601 (GenScript, USA, A01428).

602

#### 603 **TILLING lines**

604 The TILLMore population (Talame et al., 2008a, b) of 4,906 M3 families in the background of 605 the cv. Morex, mutagenized with NaN<sub>3</sub> was screened for mutations in UGT13248 (Talame et al., 606 2008a, b). We identified three non-synonymous mutations in 2,432 families screened with four 607 sets of primer pairs spanning exon 2 and the first 283 bp of exon 3. DNA samples of the 608 TILLMore resource were organized in 8-fold pools and analysed by High Resolution Melting 609 (HRM). With HRM, allelic differences are detected based on differences in melting temperature 610 using a DNA intercalating dye. A 7500 FAST Real Time- HRM- ready instrument (Applied 611 Biosystems) was used, along with Melt Doctor Amplification kit (Applied Biosystems). HRM

612 primers were designed based on the UGT13248 sequence (GenBank GU170355). Primers are listed in Supplemental Table 3. Putative mutants were confirmed by Sanger sequencing using a 613 614 TILLING fwd and TILLING rev primers (Supplemental Table 3). Three lines carrying mutations in 615 UGT13248 were identified. TILLMore #438 contained a C to T nucleotide substitution at base pair 1103 of the cDNA sequence that caused a T368I amino acid substitution of UGT13248. 616 617 TILLMore #1624 carried a G to A nucleotide substitution at bp 1105 of the cDNA sequence, 618 resulting in a S403N amino acid substitution. TILLMore #1683 carried a C to T nucleotide 619 substitution at 1208 bp, resulting in a H369Y amino acid substitution. Homozygous plants were used in the M6/ M7 generation. For Supplemental Figure 6, M6 plants were crossed with Morex 620 621 and progeny in the  $BC_1F_2$  generation was used. Sister lines that contained the homozygous 622 mutant or wild-type allele, respectively, were selected for T368I and H369Y in the  $BC_4F_2$ generation. We only selected lines with a 6-row morphology. These lines were used for Figure 623 624 6A and Supplemental Figure 11E.

625

#### 626 Full-length sequencing of UGT13248

Genomic DNA from 26 barley genotypes (Supplemental Table S1, all genotypes from Huang *et al.*, 2013) was extracted using the Plant/Fungi DNA Isolation Kit (Norgen Biotek). Sanger
sequencing was performed with primer sets designed to cover 2,477 bp of *UGT13248* genomic
sequence (Table S3). Genomic sequences of UGT13248 from 19 additional accessions were
obtained from Jayakodi *et al.* (2020) (Jayakodi et al., 2020) (Jayakodi et al., 2020). Sequences
were compared using BLAST (<u>https://blast.ncbi.nlm.nih.gov</u>).

633

#### 634 Exome capture resequencing data collection and processing

All resequencing data reported here are derived from previously published datasets, including wild, landrace, and elite barley lines reported in (Chen et al., 2022; Hemshrot et al., 2019; Kono et al., 2019; Lei et al., 2019; Mascher et al., 2013; Nice et al., 2016; Russell et al., 2016). The sequence was generated after exome capture using the barley Roche NimbleGen (Madison, WI)

639 SeqCap EZ Developer probe pools (Mascher et al., 2013).

640 For sequence alignment and quality control, we used publicly available software 641 integrated with bash scripts in the "sequence handling" workflow (Liu et al., 2022). Sequence 642 quality assessment used FastQC (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/). Adapters were trimmed using Scythe (https://github.com/vsbuffalo/scythe). Burrows-Wheeler 643 644 Aligner (BWA-MEM) (Li et al., 2009) was used to align reads to the barley reference genome 645 Morex v2 (Monat et al., 2019). Read mapping used default parameters for BWA-MEM except 646 for the following: 16 threads, minimum seed length of 8, re-seed value of 1.0, gap penalty of 8, and a minimum threshold of 85 for 100 bp PE reads or a minimum threshold of 106 for 125 bp 647 PE reads. Read mapping parameters were chosen to permit a  $\sim 2\%$  mismatch between reads 648 649 and the reference sequence; the highest estimated nucleotide diversity reported based on 650 Sanger resequencing in wild and cultivated barley (Morrell et al., 2014; Morrell et al., 2003; 651 Morrell et al., 2006). Picard v2.20.2 (https://github.com/broadinstitute/picard), was used for 652 alignment sorting, de-duplicating, and adding read groups to the read-mapped files. Read depth, and coverage estimates were generated by 'bedtools genomecov' (Quinlan and Hall, 653 654 2010). We used ~241X exome capture reads from a Morex sample (Mascher et al., 2013) to the Morex v2 reference genome empirically define regions are considered covered by exome 655 656 capture (https://github.com/MorrellLAB/captured 50x BED). These (Quinlan and Hall, 2010)

657 "cap50x" have > 50X coverage in Morex capture data

658 (<u>https://github.com/MorrellLAB/captured 50x BED</u>).

Alignment processing followed the Genome Analysis Toolkit (GATK) best practices 659 660 workflow (DePristo et al., 2011; McKenna et al., 2010). Individual sample genotype likelihoods 661 were then calculated using GATK v4.1.2 HaplotypeCaller, with a value of 0.008 per base pair passed to the "heterozygosity" option. This value is the mean estimate of coding nucleotide 662 663 sequence diversity, based on previous Sanger resequencing experiments (Caldwell et al., 2006; Morrell et al., 2014). Single sample GVCFs were merged using GenomicsDBImport, then called 664 jointly from the genotype likelihoods with the GATK tool Genotype GVCFs (McKenna et al., 665 2010). GATK's Variant Quality Score Recalibration (VQSR) was used for variant filtering. SNPs 666 667 were also filtered to include sites that fall within the "cap50x" regions, are polymorphic and bi-668 allelic with genotype quality (GQ) > 3, and have per sample depth (DP) between 5 and 78

669 (extremely high depth is likely due high copy number genomic regions collapsed in the 670 reference genome, so we take the 95th percentile of coverage as the upper cutoff). 671 Heterozygous genotypes were set to missing if the allele balance deviated more than 0.1 from 672 the expected allele balance of 0.5 (Muyas et al., 2019; Pedersen et al., 2021). Briefly, allele balance helps identify systematic or alignment errors that could result in false SNP calls. The 673 674 filtered VCF was generated as part of a larger cohort of samples with 458 of the samples used 675 for this study. Genotyping for the UGT13248 locus was extracted from the GATK-derived VCF using bcftools (Danecek et al., 2021) (https://github.com/MorrellLAB/Locus HvUGT13248). 676 UGT13248 CDS according to Morex V2 starting position is Chr5 position 330626689. 677 678

#### 679 Plant height, spike length and spike density

Height was measured as the distance from the base of the plant to the top of the spike,
excluding awns on eight plants each. Spike length was measured as the distance between the
top and bottom spikelets on five spikes. Spike density was calculated as the number of rachis
nodes per cm spike.

684

#### 685 DON plate assays, DON inoculation, and DON and D3G measurements

Barley grains were surface-sterilized for 10 minutes in a 1 % sodium hypochlorite solution 686 687 containing 0.01 % Tween 20. Grains were washed five times with sterile water and placed on ½ 688 x Murashige and Skoog (MS) medium with 0.8 % agar. Plates were kept at 4 °C for two days for stratification and then transferred to 16 h light / 8 h dark conditions at 22 °C for two to three 689 690 days. Seedlings were transferred onto ½ x MS medium containing 1 mg / L DON or mock. DON was dissolved in 70 % ethanol at 5 mg / mL and stored at -20°C. Five mg / mL DON stock 691 692 solution was added at 1:5000 ratio to medium for 1 mg / mL DON plates. Similarly, 70 % ethanol was added at 1:5000 ratio to medium for mock plates. Root length was measured daily 693 694 for six days.

For inoculation experiments, DON was diluted to 0.2  $\mu$ g /  $\mu$ L, and 10  $\mu$ L of this solution were inoculated at anthesis between the palea and lemma of each floret. We inoculated three 697 central spikelets on each side of any spike and harvested the inoculated section of the spike 698 (total of 18 spikelets). For each biological replicates spike sections from three separate spikes 699 were combined. Samples were ground in liquid nitrogen and trichothecenes extracted with 4 700 volumes per weight with a mixture of acetonitrile /water /acetic acid: 79 / 20 / 1 for 90 minutes 701 at room temperature. 500 µL per sample were dried down and resuspended in 1 mL 50 % 702 aqueous methanol, centrifuged and transferred into HPLC vials and measured using a 1290 703 Agilent UHPC system coupled to a Sciex 6500+ QTrap MS. Liquid chromatography and tandem 704 mass spectrometry details were as previously described (Fiby et al., 2021).

For Figure 6A and B, DON was measured in parts per million ( $\mu g / g$ ), by gas chromatography/mass spectrometry (Mirocha et al., 1998). Ergosterol was measured as described previously (Dong et al., 2006). Samples that were below the detection limit were set to 0 mg / L.

709

#### 710 Plant inoculation with F. graminearum

*F. graminearum* was grown in liquid CMC medium (15g / L carboxymethylcellulose (CMC), 1 g / L NH<sub>4</sub>NO<sub>3</sub>, 1 g / L KH<sub>2</sub>PO<sub>4</sub>, 0.5 g / L MgSO<sub>4</sub> x 7 H<sub>2</sub>O and 1 g / L Yeast Extract) for five days at room temperature while shaking at 150 rpm. Fungal cultures were filtered through 1-fold Miracloth, and filtrate was collected by centrifugation for 10 minutes at 2500 x g and 4 °C. Fungal spores were washed with sterile water and the concentration was adjusted to 1 x 10<sup>8</sup> macroconidia per mL.

For point inoculation experiments with *F. graminearum* strain PH-1, one central spikelet
in the middle of each side of a spike was inoculated with 10 µl *F. graminearum* (PH 1) at 1 x 10<sup>5</sup>
macroconidia / mL at anthesis in a controlled environment chamber. Inoculated spikes were
covered with a plastic bag for 48 h. FHB severity was scored at the indicated time points by
calculating the ratio of the number of infected spikelets versus the total number of spikelets.
For field experiments each genotype was planted in a 1.52 m × 0.31 m single-row plot
and inoculation experiments were performed as previously described (Huang et al., 2021). In

brief Morex, T368I, H369Y, and S403N genotypes were planted in six replicates for each

725 location (Saint Paul and Crookston) and year (2019 and 2020). The susceptible check Stander 726 and the resistant check Chevron were planted in 3 replicates for each location and year. In Saint 727 Paul, plants were spray-inoculated twice with  $1 \times 10^5$  macroconidia / mL *F. graminearum* within 728 three to four days. The first application was performed two days after heading. In Crookston, 729 plants were inoculated by spreading F. graminearum-colonized maize seed (grain spawn) onto 730 the soil surface at the four-five leaf stage and at flag-leaf emergence. Mist irrigation was used 731 from before the first inoculation and maintained until FHB severity was recorded to facilitate disease development. FHB severity was scored by calculating the ratio of the counted number 732 of infected kernels versus the total number of kernels within a spike from ten randomly 733 734 selected spikes per plot. For each plot, the ratios were averaged to obtain FHB severity for each 735 biological replicate. DON was measured on grain samples harvested from FHB nurseries in Saint Paul after threshing and cleaning. A sample of cleaned grains from each plot was measured for 736 DON accumulation, in parts per million ( $\mu g / g$ ), by gas chromatography/mass spectrometry as 737 previously described (Mirocha et al., 1998). 738

739

#### 740 Protein modeling

741 Structural models of UGT13248 were created using SWISS-MODEL (Waterhouse et al., 2018).

Protein BLAST searches were conducted within the SWISS-MODEL server. The protein sequence
 submitted for BLAST searches was

METTVTAVSGTTSSSVGHGAGGGAARVLLLPSPGAQGHTNPMLQLGRRLAYHGLRPTLVATRYVLSTTPAPGAPFDV 744 745 AAISDGFDAGGMALCPDPAEYFSRLEAVGSETLRELLLSEARAGRPVRVLVYDAHLAWARRVAQASGVAAAAFFSQPC 746 SVDVVYGELWAGRLALPATDGRALLARGVLGVELGLEDMPPFAAVPESQPAFLQVSVGQFEGLDYADDVLVNSFRDIE 747 PKEVEYMELTWRAKMVGPTLPSYYLGDGRLPSNKSYGLDLFNSEVECMDWLEKQMNSSVVLVSYGTVSNYDATQLEE 748 LGNGLCNSSKPFLWVVRSNEEHKLSEELKEKCGKIGLIVSWCPQLEVLAHRAIGCFVTHCGWNSTLEALVNGVPFVGIPH 749 WADQPTIAKYVESAWGMGVRARKNKNGCLKKEEVERCIREVMDGERKDEYKKNAMNWMQKAKEAMQEGGSSDK HVAEFATKYSSI. The template with the highest sequence identity was selected for modeling (73 750 751 % sequence identity). The model was built using the three-dimensional crystal structure of Os79 in complex with the non-reactive co-substrate UDP-2-fluoro-2-deoxy-D-glucose and 752 753 trichothecene (PDB ID: 5TMD) (Wetterhorn et al., 2016) as the search template. Structural

models were visualized in the PyMOL Molecular Graphics System, Version 1.2r3pre

755 (Schrödinger, LLC) to determine putative active site motifs. Putative ligand non-reactive mimic

756 UDP-2-fluoro-2-deoxy-D-glucose and trichothecene were docked in the active site by structural

757 alignment. Structural models of UGT13248 mutants were generated and compared in PyMOL

758 (Barber, 2021).

759

#### 760 Confocal Microscopy

A F. graminearum reporter strain 8/1 with constitutive dsRed expression (pgpdA::dsRed) and 761 762 inducible GFP expression (*pTRI5::GFP*) (Ilgen et al., 2009) was used to monitor fungal growth and trichothecene biosynthesis *in vivo*. Five  $\mu$ l of a 1 x 10<sup>5</sup> macroconidia / mL suspension was 763 764 applied to one central floret on one side of the spike and covered with clear plastic bag for 24 hours to maintain high humidity. At 14 dpi, rachises of infected barley spikes were hand-765 766 sectioned longitudinally and observed using confocal laser scanning microscopy (Nikon A1si, 767 UMN Imaging Center). Optical configuration for fluorescence detection was as previously described (Boenisch and Schäfer, 2011). Plant cell wall autofluorescence (DAPI) was excited at 768 769 406 nm and detected in the 425-475 nm range. DsRed was excited at 562 nm and detected in 770 the 575-625 nm range. Z-stacking was used to collect images at multiple focal planes. Image processing and data generation were performed using the Nikon NIS-Elements platform 771 772 (www.microscope.healthcare.nikon.com/products/software/nis-elements).

773

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#### 790 AUTHOR CONTRIBUTIONS

791

G.B. and G.J.M. designed the research. G.B., Y.H., G.H., S.H., X.L., M.Q., Y.D., and F.B. performed

research. S.M., S.S. and J.K. provided tools. G.B., Y.H., C.L., S.R.W., and P.L.M. analyzed data.

G.B. wrote the paper with input from all authors.

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#### 796 SUPPLEMENTAL MATERIALS

- 797 Supplemental Table 1: List of accessions used for full-length sequencing of UGT13248.
- 798 Supplemental Table 2: List of accessions used for exome capture sequencing analysis.
- 799 Supplemental Table 3: List of primers.
- 800 Supplemental Figure 1: Non-synonymous mutations identified in *UGT13248* in 45 barley

801 accessions.

- 802 Supplemental Figure 2: Comparison of rice Os79 and barley UGT13248.
- 803 Supplemental Figure 3: Non-synonymous mutations identified using exome capture sequencing
- 804 of 458 barley accessions.
- 805 Supplemental Figure 4: Seedling root growth upon treatment with DON.
- 806 Supplemental Figure 5: UGT13248 overexpression lines.

- Supplemental Figure 6: The wild-type *UGT13248* gene is a single dominant gene that confers
  seedling root resistance to DON.
- 809 Supplemental Figure 7: Plant height, DON and D3G measurements in field experiments.
- 810 Supplemental Figure 8: Plant Height and spike density for UGT13248 overexpression lines.
- 811 Supplemental Figure 9: Only H369Y and PI383933 plants show increased FHB severity in point
- 812 inoculation experiments.
- 813 Supplemental Figure 10: Susceptibility of PI383933 is not due to mutations in UGT13248.
- 814 Supplemental Figure 11: Disease symptoms after point inoculation with *F. graminearum*
- 815 reporter strain.
- 816 Supplemental Figure 12: *F. graminearum* reporter strain is detected in H369Y rachis tissue.
- 817 Supplemental Figure 13: Alignment of orthologous UGT sequences in rice (Os79), barley
- 818 (UGT13248), *B. distachyon* (Bradi5g03300), *Aegilops tauschii* (AET5Gv20385300) and wheat
- 819 (*Ta*UGT5D).
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- 1050

#### 1051 FIGURE LEGENDS:

#### 1052 Figure 1: UGT13248 can alleviate DON induced root growth inhibition in barley seedlings.

- 1053 A) D) Two- to three-day old barley seedlings were transferred onto 0.5 x MS medium
- 1054 containing 1 mg / L DON (DON) or equal volumes of 70 % ethanol (mock). Root length was
- 1055 measured one day later (day1) and daily thereafter for 5 additional days. Data are from two to
- 1056 four independent experiments with 4-6 seedlings each combined. Means and SEM are shown.
- 1057 Asterisks indicated significant differences at p < 0.01 using Student's T-test comparing mock
- and DON samples for each time point within each genotype. A) and B) show results for two
- 1059 independent transgenic lines that express UGT13248 under control of Zea mays Ubi-1 promoter
- 1060 (UGT+), non-transgenic sister lines (UGT-), wild-type Golden Promise and Rasmusson. C) and D)
- show plants with mutations in UGT13248 T368I, H369Y and S403N as well as wild-type Morex
- 1062 seedlings.
- 1063

#### 1064 Figure 2: Protein models of UGT13248.

Protein models based on the rice Os79 crystal structure are shown. Trichothecene is shown in
purple, the non-reactive substrate UDP-2-fluoro-2-deoxy-D-glucose in orange, the conserved
Thr299 and His38 residues in yellow and His369 and Thr368 in green. For T368I Thr at position
368 was replaced with Ile, for H369Y His at position 369 was replaced by Tyr and for S403N Ser
at position 403 was replaced by Asn. Mutated residues are shown in pink.

1070

#### 1071 Figure 3: UGT13248 catalyzes DON to D3G conversion in barley spikes.

A) Barley spikes of two sister lines one containing a *ZmUBi-1::UGT13248* transgene (UGT+) and
one without (UGT-), were inoculated with 2 μg DON into 8 spikelets per spike at anthesis.
Samples from two separate spikes were collected at the indicated time point and combined for each
biological replicate. DON and D3G were measured. Data are shown as the mean ratio of D3G to DON of
3 independent biological replicates each and SEM. Asterisks indicate significant differences between
UGT- and UGT+ at each time point at p < 0.01 using Student's T-test. B) Samples were treated and</li>
collected as described in A) for the indicated genotypes.

1079

1080 Figure 4: Mutations that reduce UGT13248 function result in increased susceptibility to 1081 *Fusarium graminearum* in the field. A) and B) Plants of the indicated genotypes were grown in 1082 the field, inoculated with F. graminearum at anthesis and scored 14 days later. For Morex, 1083 T368I, H369Y and S403N six independent rows and for Chevron and Stander three rows each per year (2019 or 2020) and environment (Saint Paul (SP) or Crookston (CR)) were used. For 1084 1085 each biological replicate 10 spikes per row were evaluated for FHB severity and combined. 1086 Mean and SEM for three to six biological replicates each are shown. Asterisks indicate 1087 statistically significant differences from wild-type Morex at p < 0.05 using Student's T-test. C) to 1088 D) Three spikes per row for each year were collected in Saint Paul at the indicated time points 1089 and combined. DON and D3G were measured for four biological replicates each. Mean and SEM 1090 for D3G to DON ratio are shown. Asterisks indicate significant differences to wild-type Morex at 1091 each time point at p < 0.01 using Student's T-test.

1092

Figure 5: UGT13248 is required for type II resistance. A) and C) photographs show
representative images of spikes for each indicated genotype 14 (A) or 21 (C) days after point
inoculation with *F. graminearum* PH-1. Scale bars are 1 cm. B) and D) Plants of the indicated
genotypes were point inoculated in one central spikelet in the middle of the spike on each side
the spike at anthesis. FHB severity was assessed at the indicated time points. 16-24 spikes each
were assessed in two independent experiments and data were combined. Mean and SEM are
plotted. Asterisks indicate statistically significant differences at p < 0.05 using Student's T-test.</li>

1100

1101 Figure 6: F. graminearum spread and DON accumulation in the rachis was increased in H369Y 1102 compared to wild-type Morex plants. A)-C) Inoculated spikes at 14 days after inoculation with F. graminearum PH-1 were separated into five equal portions and rachis and spikelets were 1103 1104 collected separately. The sections were labelled section 1 to section 5 from the basipetal 1105 portion of the spike to the most acropetal portion. Section 3 contained the inoculated spikelets. Samples from three spikes each were combined and four independent samples each were 1106 1107 collected. A) Ergosterol as a proxy for fungal growth and B) DON was measured in each sample. 1108 Means and SEM are shown. Asterisks indicate significant differences between Morex and 1109 H369Y within each section and tissue at p < 0.05 using Student's T-test. C) Graphic depiction of 1110 date shown in A) and B). Colors indicate concentration of ergosterol and DON measured.

1111

1112 Figure 7: *F. graminearum* was observed within the rachis of T368I and H369Y but not wild-

1113 type Morex plants.

A) The junction between the most basipetal part of the spike and the peduncle of H369Y, T368I
and wild-type sister lines (WT) for each allele, was imaged with confocal laser scanning
microscopy 14 days after inoculation with *F. graminearum* (strain 8/1 (gpdAp::DsRed)). Scale
bars are 200 µm. Blue color indicated autoflourescence, red color indicated fungal tissue. A 4x
objective lens was used for both wild-type and mutant plants. A zoomed in image with a 10x
objective lens was taken for both mutants. White squares indicate the region shown in higher

- 1120 magnification at 10x. B) Rachis nodes of the indicated genotypes were imaged with confocal
- 1121 laser scanning microscopy 14 days after inoculation with *F. graminearum* (strain 8/1
- 1122 (gpdAp::DsRed)). Rachis node at inoculated spikelet (inoculated) and four rachis nodes
- basipetal (basipetal) and four rachis nodes acropetal (acropetal) of the inoculated spikelet are
- shown. Scale bars are 200 µm. Blue color indicates autoflourescence, red color indicated fungal
- 1125 tissue.
- 1126
- 1127

1128 Table 1: Mutant and transgenic barley lines used in functional studies.

Line designation	Туре	Effect on UGT13248
Morex	TILLING background	none
T368I	TILLING mutant	nonsynonymous mutation
H369Y	TILLING mutant	nonsynonymous mutation
S403N	TILLING mutant	nonsynonymous mutation
Golden Promise	transformation background	none
#39003-UGT+	transformant	constitutive expression of UGT13248
#39009-UGT+	transformant	constitutive expression of UGT13248
#39003-UGT-	Non-transgenic sister line	none
#39009-UGT-	Non-transgenic sister line	none

1129



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