Genome-Wide Association Study Identifies Novel Candidate Genes for Aggressiveness, Deoxynivalenol Production, and Azole Sensitivity in Natural Field Populations of *Fusarium graminearum*

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Genome-wide association studies can identify novel genomic regions and genes that affect quantitative traits. *Fusarium* head blight is a destructive disease caused by *Fusarium graminearum* that exhibits several quantitative traits, including aggressiveness, mycotoxin production, and fungicide resistance. Restriction site-associated DNA sequencing was performed for 220 isolates of *F. graminearum*. A total of 119 isolates were phenotyped for aggressiveness and deoxynivalenol (DON) production under natural field conditions across four environments. The effective concentration of propiconazole that inhibits isolate growth in vitro by 50% was calculated for 220 strains. Approximately 29,000 single nucleotide polymorphism markers were associated to each trait, resulting in 50, 29, and 74 quantitative trait nucleotides (QTNs) that were significantly associated to aggressiveness, DON production, and propiconazole sensitivity, respectively. Approximately 41% of these QTNs caused nonsynonymous substitutions in predicted exons, while the remainder were synonymous substitutions or located in intergenic regions. Three QTNs associated with propiconazole sensitivity were significant after Bonferroni correction. These QTNs were located in genes not previously associated with azole sensitivity. The majority of the detected QTNs were located in genes with predicted regulatory functions, suggesting that nucleotide variation in regulatory genes plays a major role in the corresponding quantitative trait variation.

Genome-wide association studies (GWAS) provide a powerful tool to determine the genetic basis of quantitative traits. GWAS are commonly utilized in model eucaryotes such as humans (Frazer et al. 2009; McCarthy et al. 2008; Rietveld et al. 2013), *Drosophila* (Burke et al. 2014), and yeast (Nelson et al. 2013), but has rarely been applied to filamentous fungi. In *Neurospora crassa*, in vitro microbial communication among 24 strains was measured using confocal microscopy and was coupled with 81,614 single nucleotide polymorphisms (SNPs) to identify a gene significantly associated with the trait (Palma-Guerrero et al. 2013). A GWAS analysis utilizing 23 strains of the spruce and pine tree pathogen *Heterobasidion annosum*

identified 12 candidate virulence genes based on 33,018 SNP markers (Dalman et al. 2013).

A major limitation in all studies oriented around quantitative traits is the ability to make precise and reproducible measures of the relevant phenotype. To improve trait reproducibility, most studies measure phenotypes in highly controlled laboratory or greenhouse environments and focus on a small number of experimental strains. Because the natural field environment is prone to fluctuations that are likely to affect a quantitative trait and field populations are often composed of thousands of genetically differentiated pathogen strains, it is not surprising that candidate genes identified in laboratory studies may not show the expected effects when studied in natural field populations (Barrett and Hoekstra 2011; Xiao et al. 2009). A different approach to identify the genes underlying the traits that are most relevant in agriculture is to focus on natural, standing genetic variation in natural pathogen populations that have already been selected under field conditions, as opposed to variation that was generated through mutagenesis or selected under artificial laboratory conditions. A second way to increase the likely functional relevance of genes identified through GWAS is to generate the phenotype data using replicated trials conducted in the natural field environment.

Fusarium graminearum [teleomorph *Gibberella zeae* (Schweinitz) Petch] is a haploid, ascomycete fungus causing *Fusarium* head blight (FHB). The genome sequence revealed four chromosomes encompassing approximately 36 Mbp and 14,000 identified gene models (Cuomo et al. 2007; King et al. 2015). As for most fungi, the majority of gene models have no known function. FHB is an important disease on cereals globally, causing both significant yield losses and mycotoxin contamination. Fungicides can provide up to 70% reductions in disease when applied at or shortly after infection (Wagacha and Muthomi 2007), but fungicide resistance is likely to emerge following regular exposure (Dubos et al. 2011; Talas and McDonald 2015a). The largest group of fungicides used to control FHB is the demethylation inhibitors (DMI) that inhibit a 14 α -sterol demethylase needed to synthesize ergosterol, the primary sterol in fungal cell membranes. Point mutations in the *CYP51* gene were associated with DMI resistance in the wheat pathogen *Zymoseptoria tritici* (Brunner et al. 2008), but we could not find an association between *CYP51* sequence variation and DMI sensitivity in an earlier study (Talas and McDonald 2015a), suggesting that other genes are likely responsible for the differences in DMI sensitivity observed in the 224 field strains of *F. graminearum* included in this analysis.

Aggressiveness in *F. graminearum* was associated with two mitogen-activated protein kinase (MAPK) genes, *MGVI* and *GPMK1* (Hou et al. 2002), and one lipase-encoding gene, *FGLI*

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(Voigt et al. 2005). Other kinase genes were also reported to affect *F. graminearum* aggressiveness (Qin et al. 2015). FHB infection is usually associated with mycotoxin contamination and a close relationship was found between trichothecene production and aggressiveness (Becher et al. 2013). Although trichothecene was not essential to initiate the infection, it appears to enhance spread within colonized tissues (Boenisch and Schäfer 2011). Different genes involved in trichothecene biosynthesis are mainly clustered in a 26-kbp region called the TRI5 cluster (McCormick et al. 2004). *RAS* (GTPase-encoding) genes regulate MAPK genes and are considered essential for aggressiveness in *F. graminearum*. Disruption of *RAS2* led to slower growth on media, delayed spore germination, and a significant reduction of aggressiveness in wheat and maize (Bluhm et al. 2007).

An earlier attempt to associate nucleotide polymorphism with variation in aggressiveness and deoxynivalenol (DON) production used a candidate gene association approach. This study revealed two quantitative trait nucleotides (QTNs) within genes *MetAP1* (FGSG_08531) and *Erf2* (FGSG_01397), explaining 25.6 and 13.1% of the aggressiveness variation respectively, and one QTN within gene *Tri1* (FGSG_00071), explaining 4.4% of the variance in DON production (Talas et al. 2012b). *Tri1* encodes a cytochrome P450 monooxygenase that contributes to trichothecene biosynthesis along with the 12 biosynthetic genes located in the TRI5 cluster (Meek et al. 2003), while *Erf2* is thought to regulate subcellular localization of the RAS protein (Bartels et al. 1999; Bluhm et al. 2007). While candidate gene association can detect the quantitative effects of particular SNPs within a restricted number of previously identified genes, it cannot identify novel genes affecting a trait. A forward-genetics approach such as GWAS or QTL mapping is needed to identify novel genes.

Restriction site-associated DNA sequencing (RADseq) can provide large numbers of SNP genetic markers distributed across a genome at a relatively low cost (Baird et al. 2008). Our objective in this study was to use GWAS to identify genomic regions and candidate genes associated with aggressiveness, DON production, and propiconazole resistance in a natural population of *F. graminearum*. An important aspect of our approach was to use phenotype data collected in four distinct field environments from 119 wild-type strains sampled from a large geographical region, allowing us to identify genomic regions and candidate genes that are likely to play a significant role in aggressiveness and DON production in agroecosystems.

RESULTS

Phenotypes and genetic markers fit to the GWAS model.

The phenotypes aggressiveness, DON production, and propiconazole sensitivity followed a normal distribution (Fig. 1) with mean values of 29%, 14.8 mg kg⁻¹, and 22.2 mg l⁻¹, with heritabilities (*H*²) of 0.61, 0.67, and 0.97, respectively. The variance components analyses (Table 1) revealed significant variation among different environments, isolates, and their interaction (isolates × environment). Variance among different isolates was significantly higher than variance among different replicates of the same isolate. The variance component associated with replicates (i.e., within each environment or biological repeat) did not show significant variation for any of the traits, indicating an absence of cross infection from neighboring plots or cross-contamination among wells in microtiter plates. Aggressiveness and DON production are affected by temperature and humidity; hence, it was not surprising to find significant variation among different environments in our field experiment (Table 1).

Quality filtering resulted in 36,283 high-quality SNPs shared among 220 isolates included in the analyses. The SNP densities were equivalent across the four chromosomes and proportional to their relative sizes. Additional filters for minimum and maximum allele frequencies, maximum missing data, and linkage reduced the number to approximately 29,000 SNPs analyzed for each trait. Based on principal coordinates analysis, no population structure was detected among the isolates used in

Table 1. Analysis of variance to differentiate components contributing to phenotypic variation in aggressiveness and deoxynivalenol (DON) production

Source of variation	DF	Aggressiveness		DON production ^a	
		Estimate	Pr > F	Estimate	Pr > F
Environment (Env)	7	18.69	<0.0001	1.62	<0.0001
Replication (Rep)	1	0.99	0.0064	0.09	0.0916
Isolates (Iso) ^b	117	11.26	<0.0001	0.28	<0.0001
Env × Iso ^b	351	12.36	<0.0001	0.09	0.1661
Error	470	32.98		0.90	
<i>H</i> ²		0.61		0.67	

^a The data for this trait were BOX-COX transformed.

^b The isolates were nested according to the associated field experiment (2009/2010 and 2011/2012).

Fig. 1. The distribution of aggressiveness, deoxynivalenol (DON) production, and propiconazole sensitivity phenotypes among the *Fusarium graminearum* strains included in the genome-wide association studies. The dashed lines represent the mean value of each trait.

our GWAS population (Talas and McDonald 2015b), although it carried high genetic variation, so we did not need to correct the GWAS model for population structure. However, we corrected our GWAS model for family structure to minimize the rate of false-positive detection. In a preliminary study (Talas and McDonald 2015b), a rapid decay in linkage disequilibrium (LD) was detected over only 1,000 bp, indicating that a GWAS approach would provide high resolution in this population. Due to the nature of the field experiments used for phenotyping, different numbers of isolates were tested for different traits. The final number of isolates analyzed for aggressiveness and DON production under field conditions was 119, while 220 isolates were analyzed for propiconazole sensitivity *in vitro*. Therefore, two SNP datasets were generated independently for these two subcollections.

SNPs associated with aggressiveness, DON production, and propiconazole sensitivity.

The mixed linear model analysis generated a significance value for each SNP, using an *F* test for the null hypothesis that a

SNP has no effect on the measured trait (Zhang et al. 2010). Plots of the negative logarithmic estimates of the observed *P* values against the expected values (quantile-quantile plots) showed that the model with a kinship correction was a good fit to our datasets (Fig. 2). The correlation between the minimum allele frequency and the logarithmic *P* value indicated that the QTNs significantly associated with the traits showed a balanced allele frequency among the tested populations (Fig. 3). After applying a mixed linear model with a correction incorporating a kinship matrix within the model to reduce random genetic effects (Kang et al. 2008), we found 1,326 QTNs significantly associated ($P \leq 0.05$) with aggressiveness, 1,415 with DON production, and 1,298 with propiconazole sensitivity. The significance threshold was then adjusted by setting the false discovery rate (FDR) to a cutoff of 0.05 (Benjamini and Hochberg 1995). This reduced the number of significantly associated SNPs to 50 for aggressiveness, 29 for DON production, and 74 for propiconazole sensitivity (Fig. 4). The significance of three QTNs related to propiconazole sensitivity exceeded the Bonferroni threshold (Hochberg 1988).

Fig. 2. Quantile-quantile plots between expected and observed *P* values ($-\log_{10}$) for the traits aggressiveness, deoxynivalenol (DON) production, and propiconazole sensitivity (black dots). The straight diagonal lines represent the reference correlation.

Fig. 3. Relationships between the identified quantitative trait nucleotides for aggressiveness, deoxynivalenol production, and propiconazole sensitivity and the allele frequencies of the corresponding single nucleotide polymorphisms in the tested *Fusarium graminearum* population. MAF refers to the minimum allele frequency.

Based on the position of each SNP in the reference genome, we identified the associated genes and the position of the QTN in each gene (Tables 2, 3, 4). Most of the detected QTNs were nonsynonymous substitutions, but there were 7, 3, and 21 synonymous QTNs for aggressiveness, DON production, and propiconazole sensitivity, respectively, while 24, 12, and 23 QTNs, respectively, were located in intergenic regions. Gene annotations were based on domain homologies with other fungi using the PEDANT 3 database (Frishman et al. 2003; Riley et al. 2007). Our results were based on the annotations given in the Munich Information Center for Protein Sequences (MIPS) v.3.2; however, for some QTNs, we were able to identify an annotated gene in the reference genome sequence maintained by the Broad Institute (Cuomo et al. 2007) but not in the MIPS version of the reference genome (Wong et al. 2011). Similar inconsistencies between the Broad and MIPS annotations were reported based on RNAseq analyses of the PH-1 reference isolate (Zhao et al. 2013). A recently published update on the genome sequence of *F. graminearum* highlighted these differences (King et al. 2015).

Aggressiveness was associated with 19 nonsynonymous substitutions located in 19 genes, while seven other genes contained synonymous substitutions. The most significant QTN related to aggressiveness was located in a gene (FGSG_00355)

predicted to encode an activating protein of RAS-GTPase. Another aggressiveness-associated gene (FGSG_08010) was reported to be up-regulated during *F. graminearum* infection (Lawler et al. 2013). However, the other genes associated with aggressiveness were not identified in earlier studies of the infection process of *F. graminearum*, though domains predicted for the corresponding proteins often have a regulatory function (e.g., FGSG_00586, FGSG_13008, FGSG_01339, FGSG_02531, FGSG_05475).

Among the 17 genes associated with DON production, only FGSG_01943 was previously reported to be up-regulated during *F. graminearum* infection (Stephens et al. 2008). The remaining genes were not previously characterized in *F. graminearum*, except for their predicted function based on domain homology.

Three of the 51 genes significantly associated with propiconazole sensitivity in the GWAS exceeded the Bonferroni significance threshold (FGSG_06044, FGSG_08585, FGSG_06938). Eight genes contained domains expected to regulate transcription (FGSG_06044, FGSG_06938, FGSG_09919, FGSG_11696, FGSG_08397, FGSG_00627, FGSG_02251, FGSG_05884). One gene was related to a polyketide synthase (FGSG_04588) and six genes contained domains predicted to have a transport function (FGSG_02619, FGSG_01612, FGSG_12042, FGSG_06453, FGSG_01166, FGSG_03957). One gene encoded an ABC

Fig. 4. The positions of the analyzed RADseq single nucleotide polymorphisms across the PH-1 *Fusarium graminearum* reference genome sequence and their respective associations with aggressiveness, deoxynivalenol production, and propiconazole sensitivity. The upper solid line in each graph shows the Bonferroni threshold and the lower line shows the false discovery rate (FDR) threshold of 0.05.

transporter cassette (FGSG_09611). Three genes (FGSG_06453, FGSG_03330, FGSG_03100) contained domains of a major facilitator super family (MFS). The protein homologs of FGSG_06453 and FGSG_03330 were related to fungicide resistance in other pathogenic fungi, according to MIPS v.3.2 (Wong et al. 2011).

DISCUSSION

Many uncharacterized genes in the published draft of the *F. graminearum* genome sequence are likely to affect important agronomic traits such as aggressiveness, mycotoxin production, thermal adaptation, and fungicide resistance. Based on gene homology, Wang et al. (2011) used gene knockouts in the genome reference strain PH-1, combined with virulence tests in a greenhouse, to identify 31 protein kinase genes that affected pathogenicity. We did not identify any of these genes in our analyses. A well-structured GWAS provides a forward-genetics approach to identify novel candidate genes and regulatory sequences worthy of further molecular and biological characterization. The statistical associations between SNP markers and traits identified in this study will require additional studies oriented around functional validation in order to draw a causative conclusion. While greenhouse and growth-chamber experiments conducted in a uniform environment offer many experimental advantages, the resulting phenotypes may not accurately reflect the interactions occurring between a pathogen strain and its host under natural field conditions characterized by a continuously changing environment (e.g., with continuous fluctuations in temperature, humidity, radiation, and rainfall). Because our quantitative phenotypes were measured across many wild-type strains carrying natural genetic variation under natural field conditions in field experiments representing four different field environments, we believe that our GWAS analyses are more likely to identify chromosomal segments and corresponding genes that will be relevant in farmers' fields under real-world conditions.

Measures of trait variation.

Significant genetic variance ($P < 0.0001$) was found among isolates for aggressiveness and DON production under natural field conditions. The variance associated with different environments was significant, including differences among years, locations, and position of the experiment within the experimental field sites (Table 1). This variance likely reflects different microenvironments associated with each experimental context. Analysis of variance was used to separate the different variance components and enabled us to calculate the broad-sense heritability associated with each trait. A surprisingly high heritability was found under natural field conditions for aggressiveness ($H^2 = 0.61$) and DON production ($H^2 = 0.67$). The high heritability ($H^2 = 0.97$) associated with propiconazole sensitivity measured under tightly controlled laboratory conditions was reported earlier (Talas and McDonald 2015a).

Our GWAS analysis identified a large number of QTNs associated with each trait (110, 74, and 168 QTNs for aggressiveness, DON production, and propiconazole sensitivity, respectively) by applying the traditional FDR with a 0.10 cutoff; hence, we raised the significance threshold by applying FDR with a 0.05 cutoff, resulting in loss of around half of the QTNs for each trait. Several of the remaining significant QTNs were located in intergenic regions or could not be assigned to an annotated gene (i.e., 24, 12, and 23 QTNs for aggressiveness, DON production, and propiconazole sensitivity, respectively). These QTNs may represent regulatory elements or mis-annotated genes (Zhao et al. 2013). They may also represent SNPs that are in LD with

nearby genes or regulatory sequences that are responsible for the trait variance. Similarly, QTNs encoding synonymous substitutions may be in LD with other regions of the same gene or neighboring genes that carry nonsynonymous substitutions responsible for the observed trait variance.

QTNs associated with aggressiveness.

GWAS analysis identified a highly significant QTN explaining 24% of the measured variance in aggressiveness within the gene FGSG_00355 on chromosome 1 (Fig. 4; Table 2). This gene encodes a RAS-GTPase activating protein. The GTPase activating proteins Ira1 and Ira2 in *Saccharomyces cerevisiae* negatively regulate *RAS1* and *RAS2* (Harashima et al. 2006), which play important roles in yeast longevity (Sun et al. 1994). *RAS2* influences a MAPK cascade and cell morphology in addition to fungal pathogenicity in *Ustilago maydis* (Lee and Kronstad 2002). Similarly, *RAS2* affects growth and aggressiveness in *F. graminearum* by regulating the *Gpmk1* MAPK pathway and plays a minor role in cAMP signaling (Bluhm et al. 2007). In an earlier candidate gene association study, FGSG_08531 (*Erf2*), another gene associated with the RAS protein, explained 13% of the aggressiveness found in a subset of these *F. graminearum* isolates (Talas et al. 2012b). Hence, we associated aggressiveness with natural variation in two genes regulating the RAS protein in two different studies involving the same group of isolates. This indicates that *F. graminearum* aggressiveness in the field is significantly affected by the RAS protein, likely through its effects on the associated MAPK pathway.

Gene FGSG_08910 explained approximately 18% of the genetic variance in aggressiveness. This gene contains a CAS/CSE domain (Finn et al. 2014). CAS is involved in cellular apoptosis (programed cell death [PCD]) and proliferation (Brinkmann et al. 1996). It was reported that proper regulation of PCD is important for pathogenicity in *Botrytis cinerea* (Shlezinger et al. 2012). However, Brinkmann et al. (1996) speculated that CAS may have a regulatory function in the MAPK system, based on its intracellular colocalization on microtubules and the mitotic spindle together with the CAS putative recognition site for MAPK.

QTNs located within genes FGSG_00586, FGSG_13008, FGSG_02531, and FGSG_06075 explained 18, 15, 12, and 15% of the genetic variance, respectively. All of these genes are predicted to encode zinc binding. The first three genes contain a domain associated with zinc-finger transcription factors. However, this gene family is associated with many biological functions, including amino acid metabolism, mitosis, and meiosis. Superoxide dismutases are zinc-binding proteins that were associated with virulence in *C. albicans* (Frohner et al. 2009). Analyses of transcriptional networks in *F. graminearum* identified regulators associated with coexpression of certain genes (Lawler et al. 2013). Based on gene expression measured in microarrays, FGSG_08010 (associated with 13% of the genetic variance in aggressiveness) was shown to regulate the expression of two different groups of genes that were up-regulated during infection of a susceptible host (Lawler et al. 2013). This gene encodes a zinc finger-binding domain unique to fungi that regulates genes affecting metabolism and development. Our GWAS based on natural, standing genetic variation found in field populations supports the findings of Lawler et al. (2013). Given the relatively large number of candidate genes associated with zinc binding, we speculate that zinc plays an important role in aggressiveness of *F. graminearum*.

Overall, most of the genes associated with aggressiveness in the GWAS are involved in regulation or transport activities (Table 2). For example, FGSG_01339 encodes a TATA binding protein that is also likely to affect gene regulation. Similarly,

FGSG_05475 and FGSG_08608 are predicted to have regulatory functions.

QTNs associated with DON production.

Our GWAS identified 17 genes significantly associated with DON production. Four of these genes encoded uncharacterized

hypothetical proteins in *F. graminearum*, though these genes encode domains that may provide insight into their function. Interestingly, the most significant QTN (explaining 19% of the genetic variance) was assigned to an intergenic region on chromosome 1, consistent with a regulatory function, a misannotation, or LD with an explanatory polymorphism. A QTN explaining 12%

Table 2. Significant quantitative trait nucleotide polymorphisms in exons associated (at a false discovery rate threshold of 0.05) with aggressiveness in *Fusarium graminearum*

Chr ^a	SNP ^a	Alternative allele	Reference alleles	Position in gene	Substitution	Frequency	P value	Explained genetic variance
4	SNP24237	T	C	3518	A/V	0.19	0.000415	0.25
1	SNP00852	C	T	1781	M/T	0.11	0.000049	0.24
1	SNP01623	T	A	18	Syn (R)	0.19	0.000634	0.18
1	SNP05838	A	T	2323	S/T	0.22	0.001058	0.18
2	SNP12505	A	T	574	S/T	0.18	0.000873	0.18
2	SNP14236	G	C	641	T/R	0.08	0.002168	0.18
4	SNP25273	C	G	1300	A/P	0.14	0.001076	0.18
1	SNP07026	A	T	593	F/Y	0.16	0.000815	0.17
2	SNP16744	G	T	557	V/G	0.09	0.001043	0.17
3	SNP17675	G	A	1400	H/R	0.20	0.002099	0.17
2	SNP14402	C	T	2058	Syn (D)	0.16	0.001825	0.16
3	SNP23376	A	G	775	A/T	0.11	0.001521	0.16
1	SNP04014	G	A	1552	I/V	0.14	0.001638	0.15
3	SNP23037	G	A	5447	D/G	0.11	0.001716	0.15
3	SNP20829	T	C	2549	A/V	0.08	0.001996	0.15
3	SNP18861	T	G	1116	Q/H	0.10	0.002183	0.15
4	SNP29421	G	A	79	I/V	0.16	0.001796	0.15
1	SNP06634	A	G	515	G/D	0.12	0.0015	0.14
1	SNP06829	A	T	956	V/D	0.16	0.001962	0.14
1	SNP04746	G	A	2961	Syn (R)	0.15	0.002083	0.14
2	SNP11506	G	C	2445	Syn (G)	0.16	0.001854	0.14
2	SNP10101	C	A	1308	Q/H	0.09	0.001321	0.13
2	SNP14633	C	T	1239	Syn (N)	0.23	0.001787	0.13
1	SNP07224	G	A	348	Syn (E)	0.16	0.00215	0.12
4	SNP29198	A	G	41	R/H	0.26	0.001574	0.09
4	SNP28666	A	G	179	Syn (Stop)	0.11	0.001795	0.09

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^a Chr = chromosome; SNP = single nucleotide polymorphism.

^b General function classification. A indicates the SNP encodes a regulatory function and B indicates the SNP encodes a transport function.

Table 3. Significant quantitative nucleotide polymorphisms in exons associated (at a false discovery rate threshold of 0.05) with deoxynivalenol production in *Fusarium graminearum*

Chr ^a	SNP ^a	Alternative allele	Reference allele	Position in gene	Substitution	Frequency	P value	Explained genetic variance
1	SNP00102	C	A	831	Syn (L)	0.08	0.00078	0.15
3	SNP17537	C	T	986	V/A	0.10	0.00113	0.15
2	SNP12335	T	A	180	R/S	0.24	0.00189	0.13
4	SNP29587	T	C	1912	R/stop	0.09	0.00153	0.13
4	SNP27625	T	G	1345	E/stop	0.14	0.00196	0.13
2	SNP12939	G	C	331	Q/E	0.10	0.00059	0.12
2	SNP12986	A	C	995	A/E	0.07	0.00198	0.12
3	SNP17581	C	T	328	Y/H	0.09	0.0013	0.12
4	SNP23914	G	C	248	S/stop	0.11	0.00208	0.12
1	SNP00298	T	C	360	Syn (F)	0.14	0.0015	0.11
1	SNP05884	G	C	1696	L/V	0.13	0.00183	0.11
2	SNP12929	T	C	430	P/S	0.18	0.00123	0.11
3	SNP17742	G	A	163	I/V	0.13	0.00133	0.11
3	SNP20351	C	T	190	Stop/Q	0.10	0.00148	0.11
1	SNP06980	A	G	1268	G/D	0.08	0.00164	0.10
1	SNP00280	C	A	1421	E/A	0.09	0.00161	0.09
1	SNP03801	G	A	1788	Syn (K)	0.12	0.00213	0.08

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^a Chr = chromosome; SNP = single nucleotide polymorphism.

^b General function classification. A indicates the SNP encodes a regulatory function, B indicates the SNP encodes a transport function, and C indicates the SNP encodes a signaling function.

of the variance was in FGSG_12573 (Fig. 4), predicted to encode the enzyme 3-hydroxybutyryl-CoA dehydratase (*hbd*), a key component of the butyrate production pathway that is involved in spore formation and germination in *Clostridium difficile* (Pettit et al. 2014).

Production of type-B trichothecene in *Trichothecium roseum* is based on the trichothecene core structure EPT (12,13-

epoxytrichothec-9-ene), in which EPT undergoes hydroxylation at C-4 (–OH) followed by esterification with a butyryl group (–OR), which is then followed by a second hydroxylation at C-8 (–OH) (McCormick et al. 2011). A new trichothecene called NX-2 was recently characterized, using liquid chromatography-tandem mass spectrometry in *F. graminearum*. The nuclear magnetic resonance measurements identified NX-2

Table 2. (continued from previous page)

SNP ^a	Gene code	Molecular function	Classification ^b	Biological processes
SNP24237	FGSG_06583	ATP binding	A	
SNP00852	FGSG_00355	RAS GTPase activator	A	RAS GTPase
SNP01623	FGSG_00586	Metal ion binding	A	Regulation of transcription/DNA-dependent
SNP05838	FGSG_01935	Transferase activity	B	GPI anchor biosynthetic process
SNP12505	FGSG_08910	CAS/CSE protein family	A	Programmed cell death
SNP14236	FGSG_12420	Hypothetical protein		
SNP25273	FGSG_13008	Metal ion binding/Transcription factor activity	A	Regulation of transcription (Pig1p)
SNP07026	FGSG_12140	Metallophos 2	B	
SNP16744	FGSG_12204	Sugar transporter	B	
SNP17675	FGSG_12685	NOT2,3,5 domain	A	
SNP14402	FGSG_03620	Hypothetical protein		
SNP23376	FGSG_11179	Hydrolase activity	A	Secondary metabolism/fumarylacetoacetate hydrolase
SNP04014	FGSG_01339	DNA binding/TATA binding	A	Regulation of transcription
SNP23037	FGSG_13878	AMP binding	A	
SNP20829	FGSG_06075	Microtubule binding/Metal ion binding	A	Protein transport
SNP18861	FGSG_05475	Protein binding	A	Regulation of transcription
SNP29421	FGSG_09541	Hydrolase	A	
SNP06634	FGSG_02259	ATP adenyltransferase II	A	Phosphate metabolism
SNP06829	FGSG_02352	Acyltransferase activity	B	CIT2-citrate (si)-synthase/peroxisomal
SNP04746	FGSG_01563	Hypothetical protein		
SNP11506	FGSG_08608	PAS2/His Kinase A/Response Regulator/GAF	A	
SNP10101	FGSG_08010	Zn-cluster	A	Regulatory virulence
SNP14633	FGSG_03751	L-amino acid transport	B	Amino acid derivatives transport/major facilitator
SNP07224	FGSG_02531	Transcriptional control (zinc binding)	A	Response to stress
SNP29198	FGSG_09604	Uncharacterized		
SNP28666	FGSG_13639	Hypothetical protein		

Table 3. (continued from previous page)

SNP ^a	Gene code	Molecular function	Classification ^b	Biological processes
SNP00102	FGSG_00055	Hypothetical protein		
SNP17537	FGSG_05068	Fungal Zn(2)-Cys(6)/ATP binding	B	
SNP12335	FGSG_08847			DNA damage response
SNP29587	FGSG_09499	GTPase activator	A	Small GTPase mediated signal transduction
SNP27625	FGSG_07578	ATP binding/transferase activity	B	Phosphate metabolism
SNP12939	FGSG_12573	3-hydroxybutyryl-CoA dehydratase	A	Kinase activity
SNP12986	FGSG_02851	Fumarylacetoacetase activity	A	
SNP17581	FGSG_12679	Hypothetical protein		
SNP23914	FGSG_12927	Fungal specific transcription factor	B	
SNP00298	FGSG_11696	ATP binding	A	DNA repair
SNP05884	FGSG_01943	PAS	A/C	PAS-kinase/UV response
SNP12929	FGSG_02824	Major facilitator	B	
SNP17742	FGSG_05119	Transport facilities	B	Drug/toxin transport
SNP20351	FGSG_05945	Hypothetical protein		
SNP06980	FGSG_02441	Hydrolase activity	A	
SNP00280	FGSG_00162	Hypothetical protein	A	
SNP03801	FGSG_01273	Enzymatic activity regulation	A	Cell growth Morphogenesis

as 3 α -acetoxy-7 α ,15-dihydroxy-12,13-epoxytrichothec-9-ene, which lacks the keto group at C-8 compared with 3-acetyl (3-)-ADON (Varga et al. 2015). In other words, it appears that NX-2 is based on the EPT core structure and requires butyryl for its synthesis. The protein encoded by FGSG_12573 is essential for butyryl synthesis. It is not clear if detection of a significant QTN in FGSG_12573 is connected with the emergence of a new trichothecene variant, because our serological assay could not differentiate between DON and NX-2. But this finding indicates a need to investigate the range of trichothecene variants existing in Europe.

FGSG_01943, explaining 11% of the genetic variance for DON production, encodes a PAS kinase domain that is orthologous to a gene that coordinates glycogen synthesis in *S. cerevisiae*

(Grose et al. 2007). FGSG_01943 is differentially regulated during crown rot infection in *F. graminearum* compared with ear infection at 2 days postinoculation (Stephens et al. 2008). PAS domains sense redox changes in the electron transport system or the overall redox status and can sense environmental factors affecting cell membranes and cell metabolism (Taylor and Zhulin 1999). In *Bordetella* species that cause respiratory infection, a PAS protein was linked to a pathogenicity and toxin activator (BvgS protein) and was involved in signaling environmental changes (Taylor and Zhulin 1999). We hypothesize that FGSG_01943 may play a similar role in *F. graminearum*.

A QTN explaining 13% of the genetic variance was found in gene FGSG_09499, which encodes a GTPase activator

Table 4. Significant quantitative nucleotide polymorphisms in exons associated (at a false discovery rate threshold of 0.05) with propiconazole sensitivity in *Fusarium graminearum*

Chr ^a	SNP ^a	Alternative allele	Reference allele	Position in gene	Substitution	Frequency	P value	Explained genetic variance
2	SNP10673	T	C	62	Q/Stop	0.37	0.00000094	0.17
3	SNP19173	G	A	1221	Syn (A)	0.42	0.00000086	0.17
4	SNP23541	T	A	1194	Syn (T)	0.44	0.00000109	0.17
2	SNP11749	A	G	1898	G/E	0.25	0.00006201	0.15
1	SNP00477	G	A	294	Syn (E)	0.25	0.0000072	0.14
1	SNP07664	A	G	1008	Syn (A)	0.39	0.00005009	0.14
1	SNP06988	C	T	1104	Syn (A)	0.41	0.00005273	0.14
1	SNP04570	T	C	2908	S/P	0.43	0.00009546	0.14
4	SNP26897	G	C	2374	G/R	0.40	0.00002545	0.14
4	SNP22192	A	G	973	A/T	0.08	0.00003252	0.14
1	SNP05545	T	C	1224	Stop /Q	0.47	0.00017352	0.13
2	SNP12056	C	T	588	Syn (N)	0.10	0.00226645	0.13
3	SNP20860	T	C	2193	Syn (N)	0.33	0.00009644	0.13
3	SNP17939	G	A	1398	Syn (Q)	0.45	0.00023358	0.13
4	SNP28168	G	A	249	Syn (T)	0.43	0.00013855	0.13
4	SNP24191	T	C	156	Syn (F)	0.41	0.00028908	0.13
1	SNP06462	G	A	2024	K/R	0.27	0.00120976	0.12
2	SNP15380	G	T	5905	C/G	0.10	0.0001165	0.12
2	SNP12563	G	A	117	Syn (A)	0.45	0.00060587	0.12
3	SNP20078	A	C	2687	P/L	0.37	0.00025337	0.12
3	SNP21787	T	C	446	P/L	0.15	0.00092432	0.12
1	SNP00250	G	C	3343	L/V	0.11	0.0000995	0.11
1	SNP086283	C	A	609	E/D	0.14	0.00109387	0.11
1	SNP07127	G	C	613	P/A	0.18	0.00208512	0.11
2	SNP09492	C	T	1587	Syn (I)	0.11	0.00095633	0.11
2	SNP12939	C	A	726	R/S	0.14	0.0014147	0.11
2	SNP13914	T	C	1110	Syn (D)	0.26	0.00193982	0.11
3	SNP17476	C	T	1498	Syn (L)	0.08	0.00142706	0.11
3	SNP21737	G	A	1333	S/G	0.45	0.00224438	0.11
4	SNP21994	A	T	873	Syn(A)	0.20	0.00078041	0.11
4	SNP25548	A	G	158	D/S	0.41	0.00079858	0.11
4	SNP24970	C	A	690	Syn (V)	0.11	0.00204759	0.11
1	SNP04911	A	C	2699	A/E	0.15	0.00050843	0.10
1	SNP06206	C	G	1848	Q/H	0.08	0.00121682	0.10
2	SNP10076	G	C	252	D/E	0.14	0.00070167	0.10
2	SNP13745	A	G	1033	A/T	0.20	0.00081782	0.10
2	SNP09750	G	C	937	L/V	0.10	0.00092185	0.10
3	SNP18638	C	A	2425	Syn (R)	0.25	0.00215404	0.10
3	SNP21657	T	A	970	R/W	0.11	0.00221462	0.10
3	SNP15606	C	T	291	Syn (H)	0.08	0.00222413	0.10
4	SNP24933	T	A	754	T/S	0.12	0.00068842	0.10
1	SNP01638	C	G	2467	V/L	0.09	0.00072028	0.09
1	SNP03180	A	G	2064	Syn (T)	0.24	0.00143591	0.09
1	SNP00797	A	C	324	Syn (T)	0.26	0.00149572	0.09
1	SNP07698	G	A	3327	Syn (R)	0.08	0.00197893	0.09
3	SNP19316	T	A	644	E/V	0.19	0.00072051	0.09
3	SNP21836	A	G	178	A/T	0.12	0.00075381	0.09
4	SNP28306	G	C	553	P/A	0.26	0.00093699	0.09
4	SNP25636	C	G	678	L/F	0.10	0.00223448	0.09
4	SNP27287	G	C	649	H/D	0.11	0.00228217	0.09
1	SNP02016	G	C	2809	H/D	0.18	0.0014407	0.08

(continued on next page)

^a Chr = chromosome; SNP = single nucleotide polymorphism.

^b General function classification. A indicates the SNP encodes a regulatory function and B indicates the SNP encodes a transport function.

domain. The biological function of those genes is not clear, but other genes encoding GTPase were functionally characterized in *F. graminearum* and found to negatively (*FgRac1*) or positively (*FgRho4*) regulate DON production (Zhang et al. 2013).

QTNs were also found in genes encoding a MFS (FGSG_02824, FGSG_05119) and ATP binding domains (FGSG_05068, FGSG_11696, FGSG_07578). A deletion mutant of the *Tri12* gene that encodes an MFS in the reference PH-1 strain of *F. graminearum* produced less trichothecene than the wild type (Menke et al. 2012). Earlier studies associated ATP-binding proteins and MFS proteins with secondary metabolites (i.e., mycotoxin production) and resistance to fungicides (Coleman and Mylonakis 2009).

QTNs associated with propiconazole sensitivity.

Becher et al. (2011) used microarrays to identify 30 genes that were significantly up- or down-regulated upon exposure to azole fungicides in the reference PH-1 strain. Our analyses included 220 wild-type strains sampled from 13 field populations across Germany and coupled natural genetic variation with robust in vitro phenotypic data (Talas and McDonald 2015a) to identify candidate genes for azole sensitivity. None of the genes identified in our GWAS were identified by Becher et al. (2011), likely due to differences in experimental approaches.

The QTN in FGSG_08585 passed the Bonferroni significance threshold and explained 17% of the genetic variance in azole sensitivity (Fig. 4). This gene encodes an acyl-CoA thioesterase, a protein that plays a role in converting activated

Table 4. (continued from previous page)

SNP ^a	Gene code	Molecular function	Classification ^b	Biological process
SNP10673	FGSG_08585	Acyl-CoA thioesterase	A	Fatty acid metabolism
SNP19173	FGSG_06044	Translation initiation factor activity	A	Transcriptional control (TAF12)
SNP23541	FGSG_06938	Metal ion binding/ATP binding	A	Regulation of transcription/RNA splicing
SNP11749	FGSG_08930	Zn(2)-C6/Fungal-type DNA binding	A	
SNP00477	FGSG_00259	Hydrolase activity	A	
SNP07664	FGSG_09919	Plus-3 (DNA binding)	A	Regulation transcription
SNP06988	FGSG_02619	Transport	B	Metal binding
SNP04570	FGSG_01612	ATP binding	B	Vesicular transport
SNP26897	FGSG_09611	ABC transporter	B	
SNP22192	FGSG_12935	Zinc ion binding	A	
SNP05545	FGSG_12042	C-compound and carbohydrate metabolism	B	Cofactor transport
SNP12056	FGSG_02836	Hydrolase activity acting on glycosyl bonds	A	Metabolic process
SNP20860	FGSG_10835	Hypothetical protein		
SNP17939	FGSG_05617	Hypothetical protein		
SNP28168	FGSG_09088	C-compound and carbohydrate metabolism	A	Short-chain dehydrogenase
SNP24191	FGSG_13062	Hypothetical protein		
SNP06462	FGSG_02390	Hydrolase activity	A	Metabolic process
SNP15380	FGSG_04588	Polyketide synthase (PKS29)	A/B	Transferase activity, sexual reproduction
SNP12563	FGSG_03100	Major facilitator	B	
SNP20078	FGSG_06330	Damaged DNA binding	A	DNA repair
SNP21787	FGSG_11333	Hypothetical protein		
SNP00250	FGSG_11696	ATP binding/DNA binding	A	Translation control RNA processing
SNP086283	FGSG_10282	Transferase activity	B	heavy metal binding
SNP07127	FGSG_02666	Hydrolase activity Carboxylesterase activity	A	Polysaccharide metabolism
SNP09492	FGSG_13455	DNA binding	A	Acetylxytan esterase
SNP12939	FGSG_03330	Major facilitator	B	Transcriptional control
SNP13914	FGSG_03957	Cellular import/export transport	B	Multidrug resistance
SNP17476	FGSG_05481	Hypothetical protein		
SNP21737	FGSG_11301	Hypothetical protein		
SNP21994	FGSG_06453	Major facilitator	B	C-compound transporter/drug resistance
SNP25548	FGSG_07581	Hypothetical protein		
SNP24970	FGSG_13135	Hypothetical protein		
SNP04911	FGSG_01735	Zn(2)-C6 fungal-type DNA binding	A	
SNP06206	FGSG_02251	Metal ion binding/Kinase activity	A	Regulation of transcription
SNP10076	FGSG_08397	Regulation transcription	A	DNA binding
SNP13745	FGSG_03830	Sugar transporter/Zn(2)-C6	B	
SNP09750	FGSG_08264	Hypothetical protein		
SNP18638	FGSG_05884	ATP binding	A	TATA binding
SNP21657	FGSG_11242	Iron transporter	B	
SNP15606	FGSG_04732	Oxidoreductase activity	A	FAD binding
SNP24933	FGSG_13129	Cohesin loading factor	A	
SNP01638	FGSG_00627	Transcription repression	A	
SNP03180	FGSG_01166	Transport activity	B	
SNP00797	FGSG_00367	Hydrolase activity	A	
SNP07698	FGSG_09939	Hypothetical protein		
SNP19316	FGSG_06090	AMP binding	A	Fatty acid metabolic process
SNP21836	FGSG_11383	Ankyrin repeat	A	Kinetochore complex maturation
SNP28306	FGSG_08995	Hypothetical protein		
SNP25636	FGSG_13174	Heterokaryon incompatibility	A	
SNP27287	FGSG_09509	ATP binding	A	Hydrolase activity
SNP02016	FGSG_00740	Hydrolase activity	A	Disease virulence and defense

fatty acids into their corresponding nonesterified fatty acids. Hunt and Alexson (2002) proposed that acyl-CoA thioesterase regulates lipid metabolism by maintaining an appropriate balance of acyl-CoA, coenzyme A, and free fatty acids. Sterols and fatty acids, particularly phospholipids, are important components of fungal cell membranes (Pohl et al. 2011). By inhibiting ergosterol synthesis, azole fungicides are likely to cause significant reprogramming of lipid metabolism in fungal cells. Higher amounts of unsaturated lipids were detected, using chromatographic and mass spectrometric techniques, when *Taphrina deformans* was grown on a medium amended with propiconazole (Weete et al. 1985). The azole fluconazole has more activity against *Cryptococcus neoformans* when the intracellular fatty acid synthase is inhibited, suggesting that a block in fatty acid synthesis would positively affect an azole's impact on ergosterol synthesis and membrane formation (Chayakulkeeree et al. 2007). Our finding of a QTN in FGSG_08585 indicates that acyl-CoA thioesterase may play an important role in azole sensitivity, possibly through reprogramming the regulation of lipid metabolism in fungal cells exposed to azoles.

Two other QTNs that passed the Bonferroni threshold, each explaining 17% of the genetic variance, were found in FGSG_06044 and FGSG_06938. FGSG_06044 was predicted to encode the transcription factor TAF12 (= TFIIID) and FGSG_06938 encodes a hypothetical protein with different predicted molecular functions (metal ion binding, ATP binding, or RNA splicing), according to the MIPS database. These two genes represent completely novel contributors to antifungal resistance and provide good candidates for future studies on the mechanism of fungicide resistance in field populations of pathogenic fungi.

A DNA mismatch repair function was predicted for the FGSG_06330 protein that explained 12% of the variation in azole sensitivity (Fig. 4; Table 4). Deletion of genes responsible for DNA mismatch repair in *Candida albicans* led to an increase in susceptibility to fluconazole (Legrand et al. 2007). Studies of three antifungal agents (amphotericin, miconazole, and ciclopirox) in *C. albicans* and *S. cerevisiae* showed that DNA damage was significantly associated with antifungal activity and cell death and illustrated that blocking DNA repair could diminish fungicidal activity (Belenky et al. 2013). Our finding suggests a similar association in *F. graminearum*.

According to the Pfam database (Finn et al. 2014), FGSG_04588 (carrying a QTN explaining 12% of the genetic variance) encodes the polyketide synthase *PKS29* (Sieber et al. 2014). *PKS29*, together with a terpenoid synthetase and two methyltransferases, is significantly overexpressed 96 h after infecting wheat and remains highly expressed for 35 days after infection (Sieber et al. 2014). Gaffoor et al. (2005) reported diverse expression patterns in *F. graminearum* for *PKS* genes during plant colonization, sexual development, and mycelial growth, using gene knockouts. Unfortunately, *PKS29* was not included in the study of Gaffoor et al. (2005), so its function in *F. graminearum* remains unclear. Our findings suggest a possible relationship between polyketide synthases and azole sensitivity in *F. graminearum*.

Our GWAS analysis detected four QTNs explaining 11, 12, 11, and 14% of the genetic variance, respectively, in the genes FGSG_03330, FGSG_03100, FGSG_06453, and FGSG_09611. The first three genes encode proteins in the MFS. FGSG_09611 encodes an ATP-binding cassette (ABC) transporter. The FGSG_03330 protein showed a high homology (83%) with a multidrug resistance protein in *Staphylococcus aureus* that acts as an efflux pump (Floyd et al. 2010). The FGSG_03100 and FGSG_06453 proteins showed high homology to MFS proteins characterized in *Colletotrichum* species. Overexpression of plasma membrane efflux pumps such as MFS and ABC transporters is known to be an important mechanism of azole resistance in many fungi, including

Aspergillus nidulans, *Botrytis cinerea*, and *Zymoseptoria tritici* (Cannon et al. 2009). Our findings indicate that these proteins are also important contributors to azole resistance in natural populations of *F. graminearum*.

Our findings illustrate how GWAS has great potential to enable a forward-genetics, bioinformatics-based identification and prioritization of candidate genes affecting important traits in plant-pathogenic fungi. To the best of our knowledge, only three previous GWAS studies were conducted in fungi, including one in *Neurospora* spp. (Palma-Guerrero et al. 2013), one in *S. cerevisiae* (Muller et al. 2011), and one in the basidiomycete plant pathogen *Heterobasidion annosum* (Dalman et al. 2013). Those studies used a relatively small number of isolates (ranging from 23 to 88) and measured traits under highly controlled laboratory conditions. Ours is the first GWAS study to consider important agronomic traits measured under field conditions in an ascomycete plant pathogen.

We identified 26 genes significantly associated with aggressiveness, 17 genes associated with DON production, and 51 genes associated with propiconazole sensitivity in wild-type strains phenotyped under natural field conditions. These candidate genes were distributed randomly across the genome and explained from 8 to 24% of the genetic variance associated with these traits. Many of the candidate genes do not encode a known function or were not associated with these traits in earlier studies. Targeted functional studies of these genes will be needed to reveal the molecular mechanisms underlying their associations with the respective trait.

MATERIALS AND METHODS

Phenotyping aggressiveness, mycotoxin production, and propiconazole sensitivity.

Detailed methods for measuring aggressiveness and mycotoxin production were presented in an earlier publication (Talas et al. 2012a). Briefly, 119 isolates sampled from 13 field populations in Germany (Talas et al. 2011) were phenotyped under field conditions for aggressiveness and mycotoxin production at two different locations over two years (i.e., in four different environments total). Each single-spore isolate came from a different infected wheat spike. Seventy-one isolates were phenotyped (Talas et al. 2012b) in 2009 and 2010 at the experimental field station of the University of Hohenheim (HOH) (longitude 9° 12' 58", latitude 48° 42' 50", altitude 400 m) and at Oberer Lindenhof (OLI) (longitude 9° 18' 12", latitude 48° 28' 26", altitude 700 m). Forty-eight isolates were phenotyped in 2011 and 2012 in HOH and OLI. The experiments in 2009 and 2010 followed a split-plot design, while the experiments in 2011 and 2012 followed an incomplete block- α design. The weather conditions (temperature and precipitation) differed between HOH and OLI (Table 5). The experimental plots were arranged in a chessboard pattern of wheat and triticale plots, with each plot measuring 1 m long and 0.42 m wide and containing approximately 200 wheat heads per square meter. For all experiments, each isolate was applied to an individual plot planted to the moderately resistant wheat cultivar Taifun (KWS Lochow GMBH, Bergen, Germany) surrounded by four plots of triticale forming a tall border that limited spore movement among experimental plots. For each isolate, a 100-ml spore suspension at a concentration of 2×10^5 spores per milliliter was applied to the heads, using a sprayer with three bars of pressure at growth stage 65 of the Zadoks scale (Zadoks et al. 1974). We phenotyped each isolate in four different environments, and within each environment, the same isolate was phenotyped in two different replicates. Aggressiveness was scored as a single value, including both the number of infected heads per plot and the number of infected spikelets per head, on

a scale ranging from 0 (no infection) to 100 (each spikelet on each head infected). All wheat heads in each plot were hand-harvested, threshed, and ground to produce flour. Flour (50 g) from each plot was homogenized and extracted to quantify the amount of DON, using a commercial immunoassay kit (Ridascreen Fast Don; R-Biopharm GmbH, Darmstadt, Germany). Because the detection limit of this enzyme-linked immunosorbent assay (ELISA) was 0.23 mg kg⁻¹, smaller values were rounded to zero, resulting in a nonnormal distribution of values. Hence, we followed the suggestion of da Silva et al. (2011) to analyze the DON concentrations following a Box-Cox transformation (using SAS v.9.3) to minimize skewness or kurtosis in the data.

As a consequence of the different experimental designs and different operators involved in data collection, data were analyzed by nesting each group of isolates into its related experimental condition (i.e., *Exp1* = 2009/2010 and *Exp2* = 2011/2012). We used the following linear mixed model for the first step of data analysis:

$$y_{ijkn} = \mu + Iso_{ij} + Env_{kj} + (Iso \times Env)_{ikj} + Rep_{nk} + e_{ijkn},$$

where y_{ijkn} is the phenotype observation, μ is the intercept term, Iso_i is the genetic effect of the i th isolate in experiment j (i.e., 2009/2010 and 2011/2012), Env_{jk} is the effect of environment k in the j th experiment, $Iso \times Env$ is the interaction effect between the i th isolate in the k th environment in the j th experiment, Rep_{nk} is the effect of the n th replicate in the k th environment and e_{ijkn} is the residual error. The variance components were calculated using restricted maximum likelihood. Analyses of phenotypic data were run using SAS v.9.3 software.

The 220 isolates described earlier (Talas and McDonald 2015a), including the 119 isolates phenotyped for aggressiveness and DON production in this analysis, were tested for sensitivity to eight serial dilutions of propiconazole in vitro (i.e., 180, 120, 100, 50, 25, 10, 5, and 0.5 mg per milliliter) using three technical and two biological repeats of a microtiter plate assay as described earlier (Talas and McDonald 2015a). Briefly, each well contained 10⁵ macroconidia, ampicillin and kanamycin at 100 mg per milliliter, and different concentrations of propiconazole in synthetic nutrient broth (0.2% KH₂PO₄, 0.2% KNO₃, 0.1% MgSO₄·7H₂O, 0.1% KCl, 0.2% sucrose, wt/vol) in a total volume of 200 µl. After incubating the plates for 7 days in the dark at 24°C, the optical density (OD) of each well was measured, using an ELISA plate reader, at a wavelength of 405 nm.

The effective fungicide concentration that inhibits isolate growth by 50% (EC₅₀) was calculated for each isolate based on the OD values and according to the following nonlinear model:

$$y = \min + (\max - \min) / (1 + 10^{-(\log EC_{50} - x) \times Hillslope}),$$

where y was the OD value (in this experiment $y = 50$ for EC₅₀), $\min$ was the minimum OD value corresponding to the highest

concentration, $\max$ was the maximum OD value corresponding to the lowest concentration; x was the propiconazole concentration related to the y response, and $Hillslope$ was the steepness of the dose/response curve. EC₅₀ values of all isolates were analyzed using SAS v.9.3 software, as described earlier (Talas and McDonald 2015a).

Proc Univariate was used to verify the normal distribution of the residuals for all measured traits. Heritability (H^2) for aggressiveness, DON production, and propiconazole sensitivity was estimated as the ratio of genotypic variance to the total phenotypic variance (Fehr 1987). These analyses were performed using SAS v.9.3.

Genotyping based on RADseq.

Genomic data for 220 *F. graminearum* isolates was obtained using RADseq (Hohenlohe et al. 2010). Briefly, genomic DNA of each isolate was digested with *Pst*I and two different barcodes were ligated onto the resulting sticky ends. Afterward, different libraries were established for each barcode, were pooled into two lanes, and were sequenced using 100-bp paired-end reads on an Illumina HiSeq2000. The quality of the resulting data were checked using FASTX toolkit v.0.13. All sequences with average raw-data quality lower than 20 were discarded, using Trimmomatic v. 0.32 (Bolger et al. 2014) applied on a sliding window of 5 bp. Only sequences longer than 30 bp were retained. All alignments were performed using Bowtie2 v.2.2.0 (Langmead and Salzberg 2012). Samtools 0.1.19 (Li et al. 2009) was used to sort and index the sequences based on their barcodes. We used the published sequence of the reference isolate *F. graminearum* PH-1 (FG3, Broad Institute) to call the SNPs using the Genome Analysis Tool Kit v.3.1.1 (McKenna et al. 2010). Filtering conditions were set as minimum sequence quality = 600 and minimum sequence depth = 20 (Talas and McDonald 2015b). SNPs that did not meet these criteria were discarded using VCFtools v.3.5 (Danecek et al. 2011). These filtering steps resulted in 36,283 high quality SNPs distributed over the four chromosomes. To avoid biases in association analyses due to imbalanced allele frequencies (Massman et al. 2010), the minimum allele frequency was set to 7% and the maximum allele frequency was set to 92%. After further filtering to allow a maximum of 3.9% missing data for any SNP locus, we identified 30,797 SNPs to use for the aggressiveness and DON production traits and 28,440 SNPs for the propiconazole sensitivity trait. The SNP dataset is available online at the European Nucleotide Archive (ENA) under the study name: ena-STUDY ETH-09-10-2015-11:15:47:949-18 and the accession number PRJEB11357. Use of these data requires citation.

GWAS analyses.

A population genetic analysis based on a subset of 1,129 RADseq SNPs distributed evenly across the genome showed an

Table 5. Climatic conditions in field sites where aggressiveness and deoxynivalenol production were measured

Station ^a	Year	Average over the year			Average during the growing season ^b		
		Day temp. (°C)	Night temp. (°C)	Precipitation (mm)	Day temp. (°C)	Night temp. (°C)	Precipitation (mm)
OLI	2009	11.5	4.3	990.3	20.6	11.5	104.4
OLI	2010	9.8	3.1	989.7	20.1	11.6	126.9
OLI	2011	12.8	4.7	804.4	20.0	10.8	104.1
OLI	2012	11.2	3.8	915.8	20.9	11.5	136.4
HOH	2009	14.4	5.7	767.4	23.7	13.3	108.7
HOH	2010	12.9	5	621.7	23.6	13.7	94.4
HOH	2011	15.5	6	571.2	23.1	13.1	98.5
HOH	2012	14.7	5.8	684.2	24.2	13.7	67.3

^a OLI = Oberer Lindenhof, HOH = Hohenheim.

^b The period from first of June to end of August, including the heading and ripening stages in wheat associated with *Fusarium* head blight infection and covering the latent and infectious periods for the pathogen.

absence of population structure among the isolates included in the GWAS analyses, even though these isolates were sampled from wheat fields across Germany (Talas and McDonald 2015b). This suggests that a single meta-population of *F. graminearum* is distributed across the sampled geographical region in Germany. The same dataset exhibited a rapid decay in LD within a physical distance of 1 kb across all chromosomes, indicating a high resolution would be possible for association studies (Talas and McDonald 2015b). Consequently, no correction for population structure or LD was performed for this dataset. However, we removed highly linked SNP markers, using the PLINK v. 1.07 package (Purcell et al. 2007), resulting in a final filtered total of 29,949 SNPs analyzed for the aggressiveness and DON production traits and 28,038 SNPs analyzed for the propiconazole resistance trait.

The same mixed linear model was applied for each trait following the model:

$$y = a + bx + g + e,$$

where y is the phenotype, a is the mean term, b is the additive effect (fixed effect) of the candidate SNP to be tested for association, x is the allele effect, g is the polygenic effect (random effect), and e is the residual. The analysis was performed using the GCTA v.1.24 package (Yang et al. 2011). The polygenic effects have a distribution with a mean of zero and a covariance matrix $g = 2k\sigma_a^2$, where k is the kinship (coancestry) matrix with a pairwise estimate, and σ_a^2 is an unknown genetic variance (Zhang et al. 2010). The significance of marker-phenotype association was tested using the Wald F test with a significance threshold of $P < 0.05$. All results were adjusted using the Bonferroni correction and FDR procedure as implemented in the PLINK v. 1.07 software package (Purcell et al. 2007).

The variance explained by a single causal variant depends upon the allelic effect size and its frequency within the tested data. The genetic variance contributed by each SNP was calculated separately through the formula:

$$R^2 = 2p(1-p)a^2,$$

where p is the frequency of the causal variant and a is the effect size on an arbitrary scale (Robinson et al. 2014). The explained variance algorithm was implemented in the GABIT software package (Lipka et al. 2012). The total genetic effect of each isolate was calculated using the BLUP (best linear unbiased prediction) approach based on the combined effects of the SNPs. These results were then transformed into a BLUP solution for each SNP effect, based on a kinship matrix. R^2 refers to the genetic portion of the phenotypic variance. Values of R^2 were calculated for each QTN separately (Sun et al. 2010). The resulting P values were plotted against the expected P values, to show how well the applied model with kinship correction fit the data. The negative logarithmic values of the resulting P values were plotted against the allele frequency of each SNP to approximate the percentage of isolates sharing the same allele at each QTN. The resulting QTNs were plotted against the logarithmic value of their significance in a Manhattan plot. All figures were generated using the R package (available online).

Assignment of candidate genes and predicting their function.

The SNP position on the reference genome was combined with the chromosome number to identify genes containing significant QTNs using CLC Genomics Workbench 6 (CLC Inc., Aarhus, Denmark). The sequence of each candidate gene was translated and analyzed for any possible domains using the Pfam 29.0 database (Finn et al. 2014). In addition, each protein sequence was blasted using the National Center for

Biotechnology Information database. In parallel, the molecular and biological function of each candidate gene was investigated using the MIPS database for *F. graminearum*. We focused our attention on genes with well-known functions in *F. graminearum* and genes with clearly identified domains possessing high homology with other pathogenic fungi.

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AUTHOR-RECOMMENDED INTERNET RESOURCE

The R Project for Statistical Computing: <http://www.R-project.org>