A Genome-Wide Association Study To Understand the Effect of Fusarium verticillioides Infection on Seedlings of a Maize Diversity Panel

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ABSTRACT  Fusarium verticillioides, which causes ear, kernel and stem rots, has been reported as the most prevalent species on maize worldwide. Kernel infection by F. verticillioides results in reduced seed yield and quality as well as fumonisins contamination, and may affect seedling traits like germination rate, entire plant seedling length and weight. Maize resistance to Fusarium is a quantitative and complex trait controlled by numerous genes with small effects. In the present work, a Genome Wide Association Study (GWAS) of traits related to Fusarium seedling rot was carried out in 230 lines of a maize association population using 226,446 SNP markers. Phenotypes were scored on artificially infected kernels applying the rolled towel assay screening method and three traits related to disease response were measured in inoculated and not-inoculated seedlings: plant seedling length (PL), plant seedling weight (PW) and germination rate (GERM). Overall, GWAS resulted in 42 SNPs significantly associated with the examined traits. Two and eleven SNPs were associated with PL in inoculated and not-inoculated samples, respectively. Additionally, six and one SNPs were associated with PW and GERM traits in not-inoculated kernels, and further nine and thirteen SNPs were associated to the same traits in inoculated kernels. Five genes containing the significant SNPs or physically close to them were proposed for Fusarium resistance, and 18 out of 25 genes containing or adjacent to significant SNPs identified by GWAS in the current research co-localized within QTL regions previously reported for resistance to Fusarium seed rot, Fusarium ear rot and fumonisin accumulation. Furthermore, linkage disequilibrium analysis revealed an additional gene not directly observed by GWAS analysis. These findings could aid to better understand the complex interaction between maize and F. verticillioides.

Maize plants are attacked by several Fusarium species responsible for diseases such as root rot, stalk rot, seedling blight and ear rot, but Fusarium verticillioides (Sacc.) Nirenberg (synonyms Fusarium moniliforme, Gibberella fujikuroi MP-A or Gibberella moniliformis) (Bömke et al. 2008) has been reported as the most widespread fungal pathogen of maize worldwide (Leslie 1991; Danielsen et al. 1998; Chulze et al. 2000; Rahjoo et al. 2008). Fusarium infection can result in depleted seed yield and quality as well as fumonisins contamination. This fungus produces a wide range of mycotoxins that include fusaric acid, fusarins, and fumonisins (Desjardins and Proctor 2001). Fumonisins are the most prevalent (Munkvold 2003) and have been associated with esophageal cancer in humans, pulmonary edema in pigs, leukoencephalomalacia in horses and cancer-promoting activity in rats (Marasas 2001; Voss et al. 2002; Murillo-Williams and Munkvold 2008). Moreover, F. verticillioides can establish asymptptomatically inside maize plants and kernels, impairing the possibility to easily detect affected grains (Munkvold and Desjardins 1997; Munkvold et al. 1997; Wilke et al. 2007).

F. verticillioides has available many infection pathways to reach plant tissues (Battilani et al. 2003), including the transmission from soil or the presence of seed borne F. verticillioides strains in all the plant tissues such as ears and kernels (Munkvold et al. 1997; Mesterházy et al. 2012). The frequency of fungal strain transmission from the seed depends on fungal characteristics, maize genotypes and
environmental conditions (Wilke et al. 2007). Even though the complexity of maize–F. verticillioides interaction and the influence of seedling transmitted fungal strains on stalk, ear, and kernel rots as well as the fumonisin production was previously shown (Munkvold et al. 1997; Yates et al. 2003; Wilke et al. 2007; Williams et al. 2007), the potential genetic resistance to Fusarium seedling rot has not been widely examined yet.

Kernel infection by F. verticillioides can reduce seed germination of maize as well as vigor at different growth stages (Machado et al. 2013). The effect of F. verticillioides on the development of maize seedlings/plants under controlled conditions was previously evaluated (Machado et al. 2013). All traits measured (plant population stand, speed of seedling emergence, and height/weight of emerged plants) were negatively affected by the increasing fungal amounts present in the seeds (Machado et al. 2013). Recently, a genome-wide association study (GWAS) using artificially inoculated maize kernels allowed the identification of several SNPs and candidate genes significantly associated with Fusarium resistance at the seedling stage (Stagnati et al. 2019).

The cultivation of resistant germplasm is an efficient way to reduce yield loss and mycotoxin contamination, however, no maize genotypes immune to Fusarium infection have been identified and many commercial hybrids have less resistance than desired (Lanubile et al. 2010; Zila et al. 2013). Resistance to F. verticillioides in maize, which was mainly focused on Fusarium ear rot (FER), has been studied in different surveys based on artificial inoculation in field (Drepper and Renfro 1990; Reid and Zhu 2005; Reid et al., 2009; Lanubile et al. 2011, 2017; Zila et al. 2013; Maschietto et al. 2017), greenhouse (Danielsen et al. 1998; Lanubile et al. 2013, 2014a; Maschietto et al. 2016) and laboratory trials (Ju et al. 2017; Atabaki et al. 2018). Moreover, in order to develop genetically resistant germplasm to Fusarium researchers carried out Quantitative Trait Locus (QTL) mapping analysis and GWAS by testing several distinct population structures (McMullen et al. 2009; Yang et al. 2010; Zila et al. 2013, 2014; Warburton et al. 2013; Chen et al. 2016; Ju et al. 2017; Maschietto et al. 2017; Gaikpa and Miedaner 2019; Samayo et al. 2019; Septiani et al. 2019).

In the present study, the Rolled Towel Assay (RTA) screening method was applied to a maize association panel (Flint-Garcia et al. 2005) for evaluating the effect of F. verticillioides infection on the vegetative response of maize seedlings, considering plant seedling length (PL), plant seedling weight (PW) and germination rate (GERM). The panel was previously analyzed for resistance to Fusarium infection of seedlings (FIS; Stagnati et al. 2019). In order to identify SNPs putatively associated with PL, PW and GERM, with and without kernel infection by F. verticillioides, GWAS and linkage disequilibrium (LD) analysis were performed.

MATERIALS AND METHODS

Maize germplasm, kernel inoculation and phenotyping
The maize core diversity panel, sometimes referred as the “Goodman” association panel (Flint-Garcia et al. 2005; Zila et al. 2013) was used in
this work. Seeds were retrieved from USDA-ARS-NCRPIS (Iowa State University, Regional Plant Introduction Station, Ames, Iowa, United States, 50011-1170). The panel was previously evaluated for response to F. verticillioides inoculation (FIS) by Stagnati and co-workers (2019). In that work, data about seedling germination were exclusively used in order to remove lines with <50% germination percentage (Stagnati et al., 2019). Accordingly, only 230 out of 302 inbred lines of the population having sufficient germination rate were screened and considered for further analysis in the present study (Table S1).

To artificially infect mature kernels and evaluate the effect of F. verticillioides on some vegetative traits of maize seedlings, the Rolled Towel Assay (RTA) phenotyping method was used (Ellis et al. 2011; Lanubile et al. 2015; Stagnati et al. 2019). For each inbred line, seeds with similar size and shape, and without visible damage, were selected. To reduce as much as possible the presence of contaminating fungi, seeds were surface-sterilized as previously described (Stagnati et al. 2019). RTA were prepared using 10 seeds for each inbred per towel of moistened germinating paper (Anchor Paper, Saint Paul, MN). Kernels were inoculated on the embryo side near the pedicel with 100 μl of 1x10^6 conidial suspension of F. verticillioides ITEM10027 (MPVP 294) and sterilized distilled water for treated and control towels, respectively. Incubation was performed for 7 days in the dark at 25°C as previously reported (Bernardi et al. 2018; Stagnati et al. 2019).

For each seedling, plant length (PL) from the tip to the end of the longest root, and fresh plant weight (PW) of maize genotypes in control (C) and inoculated (I) conditions were calculated. Germination percentage was evaluated as reported by Stagnati et al. (2019).

**Association analysis, candidate gene discovery and linkage disequilibrium analysis**

The ZeaGBSv2.7 set of SNP derived from Genotyping By Sequencing and available at Panzea (www.panzea.org) was used. Monomorphic and multiallelic SNPs and INDELs were discarded. Imputation was performed using the software Beagle 4.1 (Browning and Browning 2016).

![Figure 2](image-url)  
**Figure 2** Correlation among disease severity (SEV; Stagnati et al. 2019), plant length (PL), plant weight (PW), germination percentage (G) traits measured in the control (C) or inoculated (I) plants. The distribution of each trait is shown on the diagonal. On the bottom of the diagonal the bivariate scatter plots with a fitted line are displayed. On the top of the diagonal the value of the correlation plus the significance level are reported as stars (** p-value 0.001).
Heterozygous were set as missing data and removed the SNPs if >20% of missing data and minor allele frequency (MAF) <5%. After the final imputation, a set of 226,446 SNPs was used for association analysis.

Marker pairs with genotypic correlation higher than $r = 0.5$ were pruned according to a linkage disequilibrium-based approach using the software Plink v1.07 (Purcell et al. 2007). A subset of around 100kb SNPs was obtained to compute the additive relationship matrix (K) for the 230 inbred using TASSEL v5.2.25 (Bradbury et al. 2007).

Genome Wide Association Analysis (GWAS) was performed in Tassel (version 5.2.25). The mixed linear model (MLM) fitted by Tassel was:

$$y = X\beta + Zu + e$$

Where $y$ is the vector of phenotypes, $\beta$ is a vector of the overall mean and the fixed effect estimate of an individual SNP, $u$ is a vector of random line additive genetic effects, $X$ and $Z$ represent incidence matrices, and $e$ is a vector of random residuals. Variance of random line effects was modeled as $\text{Var}(u) = K\sigma^2_u$, where $\sigma^2_u$ is the estimated additive polygenic variance. The optimum compression level option (compressed MLM) was used (Zhang et al. 2010).

To identify robust SNP associations, a resampling procedure was performed. In each of 100 data resamplings, a random sample of 80% of inbred lines was selected from the population, GWAS was performed on the subset of lines. Only SNP markers determined as significant at $P < 1 \times 10^{-4}$ within at least 30% of data subsamples, i.e., a resample model inclusion probability (RMIP) threshold of 0.30, were considered as significant (Samayoa et al. 2015; Stagnati et al. 2019). Data manipulations and visualizations were performed using R software (R Core Team 2016).

Genes containing or close to associated SNPs were detected using Maize GDB genome browser using the B73 RefGen_V3 as the reference genome to place SNPs and genes avoiding low confidence genes and transposable elements. The function and conserved domains of proteins encoded by genes identified were retrieved submitting sequences to NCBI-protein BLAST (https://blast.ncbi.nlm.nih.gov) and restricting the search to Zea mays (taxid:4577). For SNPs positioned inside genes the possible effect on protein sequence was inquired. Introns and translated regions were identified using GeneWise online tool (http://www.ebi.ac.uk), coding sequence were translated using ExPaSy (http://web.expasy.org/translate) and amino-acid variations were detected aligning the reference protein retrieved from MaizeGDB, and the SNP derived protein using MultAlin (http://multalin.toulouse.inra.fr).

Linkage disequilibrium (LD) measures ($r^2$) were estimated using TASSEL version 5.0 between each significant SNP and other SNPs within a surrounding window of 60 adjacent SNPs (Samayoa et al. 2015; Stagnati et al. 2019). For clusters of tightly linked significant SNPs, LD analysis was computed for one of them; positions were considered linked to significant SNPs if $r^2 > 0.5$ and the distance between the position and the SNP resulting from GWAS was greater than 2 Kb.

### Data availability
All supporting data are included as supplemental files and are available at Figshare: https://figshare.com/s/54bb47a6abf418c859b9. Table S1. (Excel file) List of germplasm and phenotypic values. Table S2. (DOCX file) Table of LD analysis.

### RESULTS AND DISCUSSION

#### Phenotypic data
Seed germination was affected in different ways by artificial inoculation (Figure 1; Table S1). In 59 cases, germination rate were higher in the inoculated towels, whereas in 87 lines the germination was negatively affected by the fungus. Similar numbers were also observed in 84 lines without and with infection. Figure 1 illustrates the change in germination due to inoculation for 20 inbreds with the most extreme reactions after inoculation. The germination of inbreds I29 and CML238 was reduced by 70% resulting in 10 and 20%, respectively, after inoculation; germination of Il677a was reduced from 50 to 10% by the inoculation. I29 (a popcorn) and Il677a (a sweet corn) were the inbreds with the lowest germination rate after inoculation and both showed increased disease severity values following inoculation (Stagnati et al. 2019).

The ability of *F. verticillioides* to influence seed germination and vigor in maize was previously explored. Some studies reported that the fungus can condition host fitness promoting growth and inducing defense mechanisms such as lignin deposition in the cell wall (Yates et al. 1997). Seedlings grown from seeds inoculated with *F. verticillioides* showed reduced growth at early stages of infection, but by 21 days after planting the inoculated plants adjusted or overtook the mock plants in shoot diameter, root growth and dry weight (Yates et al. 1997). Additionally, previous reports in literature highlighted how maize seed lots with high incidence of this fungus (inoculum potential) underwent little or no reduction in germination or seedling growth, while others were seriously compromised by the fungus (Munkvold et al. 1997; Oren et al. 2003; Machado et al. 2013). Beside the fungal inoculum potential in a seed lot, further sources of variation in seedling germination should be taken in account, such as the genetic nature of the several inbred employed in this study that can have influenced the establishment of the maize-fungal association, determining even a beneficial effect to some hosting lines.

No significant correlations were observed in non-inoculated assays between either PL_C or PW_C and the FIS severity (Sev_C), previously evaluated in Stagnati et al. (2019), whereas a moderately negative correlation was found for the germination percentage (G_C; Figure 2).
As expected, after *F. verticillioides* infection, PL_I, PW_I and G_I showed significant negative correlations with Sev_I (*r* = -0.75, -0.55 and -0.78 for PL_I, PW_I and G_I, respectively; Figure 2). In line with these achievements, previous studies reported that *F. verticillioides* infection diminished germination of maize kernels and hampered seedling growth and vigor (Van Wyk et al. 1988; Septiani et al. 2019).

Furthermore, seedling traits (length, weight and germination) of both inoculated and control assays were significantly correlated each other (Figure 2). This finding could be explained by the inherent inbred line characteristics like seed size, weight and seedling growth vigor.

**Association analysis and SNP discovery**

GWAS analysis resulted in 42 SNPs, distributed across all chromosomes, significantly associated with the traits examined. 35 SNPs were located in 21 genes, while the remaining 7 were intergenic, but within 1.1 kb of known genes (Tables 1 and 2; Figure 3). A total of 25 genes were identified as containing or close to SNPs significantly associated with at least one of the measured traits. Six SNPs were in common between two traits each, i.e., four SNPs for PL_I and PW_C, one SNP for PL_I and GERM_I and one SNP for PL_C and PW_C.

For the trait PL_C only two SNP associations were discovered, both on chromosome 4, but inside two different genes (Figure 3A). Eleven SNPs were found associated with the trait PL_I (Figure 3B): including a cluster of four SNPs in or near the same gene on chromosome 1, one SNP on chromosome 3, three SNPs inside the same gene on chromosome 5 and further three SNPs on chromosome 9. Five SNPs in or near two different genes on chromosome 1 and a SNP on chromosome 4 were associated with PW_C (Figure 3C). For PW_I an associated SNP was found in a gene on chromosome 1, seven SNPs were in a gene on chromosome 3 and one SNP was in a gene on chromosome 5 (Figure 3D).

![Table 2 Marker name, allelic variants (SNP), number of lines carrying each allelic variant (OBS), additive effect, p-values for the association between the SNP and the phenotype, resample model inclusion probability (RMIP) and proportion of the phenotypic variance associated with the SNP (R^2) referred to the traits PL_C, PL_I, PW_C, PW_I, GERM_C and GERM_I](https://example.com/table2.png)
For the trait GERM_C only one significant SNP was identified and it was inside a gene on chromosome 8 (Figure 3E). For GERM_I thirteen SNPs were observed (Figure 3F). One is located in a gene on chromosome 1, two SNPs highlighted two genes on chromosome 2 and three SNPs were found on chromosome 3 associated with 3 genes. On chromosome 4, 4 SNPs were identified inside the same number of genes; on chromosome 6 a SNP highlighted one gene and on chromosome 8 two SNPs, one inside and one close by two different genes.

The highest number of genes was identified on chromosome 5, while chromosome 6 had only one gene associated to disease traits. Genes highlighted were generally at the end of the chromosome than in centromere or pericentromeric regions. No SNPs on chromosomes 7 and 10 were associated to traits.

**Genes associated with PL, PW and GERM traits**

Table 3 reports all genes containing or close to SNP markers deriving from GWAS and significantly associated to the traits length, weight and germination in control and inoculated seedlings. A SNP (S4_768454) was found inside the gene GRMZM2G058394 and associated with traits PL_C and PW_C. This gene encodes a putative thaumatin like-protein (TLP). TLPs are implicated in host defense and in various developmental processes. These genes are expressed in response to environmental perturbations, drought, cold and pathogen attacks. Therefore, TLPs are classified as pathogenesis related protein (PR) (Marchler-Bauer et al. 2015). TLPs are universal in plants. The antifungal activity of TLPs is well reported (Liu et al. 2010; Sharma et al. 2020) and for some of them the binding activity to β-1,3-D-glucanase is described. β-1,3-D-glucan is a key component of fungal cell wall and the activity of this enzyme is important in the breakdown of fungal cell (Liu et al. 2010; Sharma et al. 2020). The TLP family also includes a xylanase inhibitor (Liu et al. 2010; Fierens et al. 2007); considering that xylan is an important component of plant cell wall, the xylanase activity of TLPs is one of the mechanisms by which these proteins are involved in plant-defense mechanism (Liu et al. 2010). In maize ears and silks infected by

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**Figure 3** Manhattan plots of the traits PL_C (A), PL_I (B), PW_C (C), PW_I (D), GERM_C (E) and GERM_I (F). SNPs significantly associated to the traits are indicated by green dots.
Table 3: Genes containing or near SNPs significantly associated with the traits PL, PW and GERM in control and *Fusarium* inoculated seedlings. For each marker, the genomic position (pos_AGP3), the surrounding or adjacent gene, the type and the conserved domain of the encoded protein (where available) and the amino-acid variation caused by the SNP are reported.

| N° | Gene_id     | Strand | pos_AGP3 | Description                                                                 | Conserved domains                                                                 | Genic | Amino-acid |
|----|-------------|--------|----------|------------------------------------------------------------------------------|----------------------------------------------------------------------------------|-------|------------|
| 1  | GRMZM5G5851807 | +      | 237936341| Putative DNA-directed RNA polymerase family protein                           | PRK13042, superantigen-like protein, DUF3223, SPT5 elongation factor              | TRUE  | E_UTR      |
| 2-19| GRMZM2G058394  | +      | 768454   | Putative thaumatin domain family protein                                      | Glycoside hydrolase family 64                                                  | TRUE  | No change  |
| 3-15| GRMZM2G087186  | —      | 45520333 | Pyruvate decarboxylase isozyme 3                                              | Pyruvate decarboxylase domains                                                  | TRUE  | No change  |
| 4-16| GRMZM2G087186  | —      | 45520465 | Putative AP2/EREBP transcription factor superfamily protein                   | AP2 domain                                                                      | TRUE  | No change (T1), E_UTR(T2) |
| 5-17| GRMZM2G073484  | +      | 45520473 | Glycoside hydrolase family 64                                                  | No change                                                                       | TRUE  | E_UTR      |
| 6-18| GRMZM2G087186  | —      | 45520608 | Putative ubiquitin-conjugating enzyme family                                  | UBCc superfamily                                                               | TRUE  | NA         |
| 7-33| GRMZM2G141638  | +      | 166839392| Putative AP2/EREBP transcription factor superfamily protein                   | Putative DNA-directed RNA polymerase family protein                             | TRUE  | E_UTR      |
| 8   | GRMZM2G027232  | +      | 30775149 | 50S ribosomal protein L11;                                                     | Ribosomal protein                                                               | TRUE  | E_UTR      |
| 9   | GRMZM2G027232  | —      | 30775150 | Putative ubiquitin-conjugating enzyme family                                  | RABGAP_TBC domain GTPase activator protein in yeast                           | TRUE  | E_UTR      |
| 10  | GRMZM2G031370  | +      | 10643186 | Uncharacterized protein                                                        | TRUE                                                                             | TRUE  | E_UTR      |
| 11  | GRMZM2G058162  | —      | 10571097 | Putative ubiquitin-conjugating enzyme family                                  | TRUE                                                                             | TRUE  | E_UTR      |
| 12  | GRMZM2G338376  | —      | 10848237 | Uncharacterized protein                                                        | TRUE                                                                             | TRUE  | Intron     |
| 13  | GRMZM2G012224  | +      | 29053916 | Putative uncharacterized protein                                               | TRUE                                                                             | TRUE  | Intron     |
| 14  | GRMZM2G012224  | —      | 29053916 | Putative uncharacterized protein                                               | TRUE                                                                             | TRUE  | Intron     |
| 15  | GRMZM2G012224  | —      | 29053916 | Putative uncharacterized protein                                               | TRUE                                                                             | TRUE  | Intron     |
| 16  | GRMZM2G012224  | —      | 29053916 | Putative uncharacterized protein                                               | TRUE                                                                             | TRUE  | Intron     |
| 17  | GRMZM2G012224  | —      | 29053916 | Putative uncharacterized protein                                               | TRUE                                                                             | TRUE  | Intron     |
| 18  | GRMZM2G012224  | —      | 29053916 | Putative uncharacterized protein                                               | TRUE                                                                             | TRUE  | Intron     |
| 19  | GRMZM2G012224  | —      | 29053916 | Putative uncharacterized protein                                               | TRUE                                                                             | TRUE  | Intron     |
| 20  | GRMZM2G012224  | —      | 29053916 | Putative uncharacterized protein                                               | TRUE                                                                             | TRUE  | Intron     |
| 21  | GRMZM2G012224  | —      | 29053916 | Putative uncharacterized protein                                               | TRUE                                                                             | TRUE  | Intron     |
| 22  | GRMZM2G012224  | —      | 29053916 | Putative uncharacterized protein                                               | TRUE                                                                             | TRUE  | Intron     |
| 23  | GRMZM2G012224  | —      | 29053916 | Putative uncharacterized protein                                               | TRUE                                                                             | TRUE  | Intron     |
| 24  | GRMZM2G012224  | —      | 29053916 | Putative uncharacterized protein                                               | TRUE                                                                             | TRUE  | Intron     |
| 25  | GRMZM2G012224  | —      | 29053916 | Putative uncharacterized protein                                               | TRUE                                                                             | TRUE  | Intron     |
| 26  | GRMZM2G012224  | —      | 29053916 | Putative uncharacterized protein                                               | TRUE                                                                             | TRUE  | Intron     |
| 27  | GRMZM2G012224  | —      | 29053916 | Putative uncharacterized protein                                               | TRUE                                                                             | TRUE  | Intron     |
| 28  | GRMZM2G012224  | —      | 29053916 | Putative uncharacterized protein                                               | TRUE                                                                             | TRUE  | Intron     |
| 29  | GRMZM2G012224  | —      | 29053916 | Putative uncharacterized protein                                               | TRUE                                                                             | TRUE  | Intron     |
| 30  | GRMZM2G012224  | —      | 29053916 | Putative uncharacterized protein                                               | TRUE                                                                             | TRUE  | Intron     |
| 31  | GRMZM2G012224  | —      | 29053916 | Putative uncharacterized protein                                               | TRUE                                                                             | TRUE  | Intron     |
| 32  | GRMZM2G012224  | —      | 29053916 | Putative uncharacterized protein                                               | TRUE                                                                             | TRUE  | Intron     |
| 33  | GRMZM2G012224  | —      | 29053916 | Putative uncharacterized protein                                               | TRUE                                                                             | TRUE  | Intron     |
| 34  | GRMZM2G012224  | —      | 29053916 | Putative uncharacterized protein                                               | TRUE                                                                             | TRUE  | Intron     |
| 35  | GRMZM2G012224  | —      | 29053916 | Putative uncharacterized protein                                               | TRUE                                                                             | TRUE  | Intron     |
| 36  | GRMZM2G012224  | —      | 29053916 | Putative uncharacterized protein                                               | TRUE                                                                             | TRUE  | Intron     |
| 37  | GRMZM2G012224  | —      | 29053916 | Putative uncharacterized protein                                               | TRUE                                                                             | TRUE  | Intron     |
| 38  | GRMZM2G012224  | —      | 29053916 | Putative uncharacterized protein                                               | TRUE                                                                             | TRUE  | Intron     |
| 39  | GRMZM2G012224  | —      | 29053916 | Putative uncharacterized protein                                               | TRUE                                                                             | TRUE  | Intron     |
| 40  | GRMZM2G012224  | —      | 29053916 | Putative uncharacterized protein                                               | TRUE                                                                             | TRUE  | Intron     |
| 41  | GRMZM2G012224  | —      | 29053916 | Putative uncharacterized protein                                               | TRUE                                                                             | TRUE  | Intron     |
| 42  | GRMZM2G012224  | —      | 29053916 | Putative uncharacterized protein                                               | TRUE                                                                             | TRUE  | Intron     |

1: indicates if the gene is on the forward (+) or reverse (-) strand. 2: indicates if the marker is inside (TRUE) a gene, otherwise the distance from the closest gene is reported. 3: indicate the amino acid variation (reference amino acid/position of variation/new amino acid), SNPs located in un-translated regions are reported (E_UTR); if the SNP had different consequences on different transcripts, of the same gene, that of interest is reported, NA means that the marker is not inside the gene.
F. verticillioides, thaumatin gene had lower expression ratios in resistant lines compared to susceptible ones. In contrast, kernels of the resistant lines showed higher gene expression before fungal infection, highlighting the key role of constitutive resistance in limiting pathogen attack (Lanubile et al. 2010, 2012a,b; Maschietto et al. 2016). The SNP found in this gene did not cause an amino-acid change in the protein.

A SNP in the gene GRMZM2G338376, which encodes a protein kinase with domains common to the receptor like kinase (RLK), was associated with PL_I. RLKs are surface transmembrane receptors able to perceive different signals and the presence of pathogens. The RLK family includes the BAK1 that is involved in brassinosteroid-mediated plant development. Interestingly, in previous studies this gene was highly expressed in maize coleoptile at V1 stage (Sekhon et al. 2011) and presented a strong modulation in resistant ears at 72 hr after F. verticillioides infection (Lanubile et al. 2014a; Wang et al. 2016). The SNP found in this gene did not cause an amino-acid change in the protein.

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The SNP S5_31445260 associated with PW_I encodes the glycine rich protein 3. These proteins are key component of plant cell wall, and their expression was described in response to lesions caused by biotic and abiotic factors, in defense against Pseudomonas syringae, and two glycine rich peptides isolates from Capsella bursa-pastoris were active against several bacteria and fungi (Mangeon et al. 2010).

Additional SNPs associated with GERM_I were identified in the GRMZM2G028568 and GRMZM2G125432 genes. GRMZM2G028568 encodes an uncharacterized protein with the Serine/threonine kinase domain, involved in plant resistance, and the wall-associated receptor kinase galacturonan-binding, which is an extracellular part of the serine/threonine kinase binding cell-wall pectins. This domain is responsible of the phosphorylation of Serine-Threonine residues and implicated in plant resistance (Afzal et al. 2008). GRMZM2G125432 encodes a protein with Pre-SET and SET domains. Pre-SET domain stabilizes SET domain, which is a lysine methyltransferase involved in protein-protein interactions (Thorstensen et al. 2011). A group of SET domains have been classified as PR proteins.

**Linkage disequilibrium analysis**

Linkage disequilibrium (LD) analysis was performed in a window of 60 adjacent SNPs on the same chromosome to identify correlations between markers. Genome-wide, 4 SNPs or groups of SNPs were detected in LD of $r^2$ greater than or equal to 0.5 to significantly associated markers (Table S2). Generally, for each trait-associated SNP examined, from 1 to 3 SNPs were detected to be in LD $r^2 > 0.5$.

Regarding the trait PW_I, only the gene GRMZM5G800586 was found both by GWAS and LD analysis; this gene encodes a protein of unknown function.
For the trait GERM_I, three positions were found in LD in the gene GRMZM2G497710 that was also identified by GWAS; also GRMZM2G125432 already reported as containing a SNP was the closest to a position found by LD analysis.

Genome-wide, three genes were identified by both analyses (GWAS and LD); while LD analysis exclusively revealed only one new gene.

**Comparison between genes detected by GWAS and QTL for resistance to Fusarium**

Since QTL often refer to chromosomal regions that may span several Mb of the genome, in which several genes may be located, genes overlapping previously reported QTL involved in resistance to FER, fumonisin accumulation, *Fusarium* seed rot (FSR) and seedling traits, including seedling length, weight and FIS, were investigated (Figures 4-6). Genes containing or adjacent to significant SNPs identified by GWAS analysis were located on chromosomes according to their physical position. In parallel, known QTL for the traits mentioned above were located on chromosomes according to ‘bin’ positions (Zila et al. 2013; Lanubile et al. 2014b; Ju et al. 2017; Maschietto et al. 2017; Septiani et al. 2019).

On chromosome 1, genes were well distributed and associated for all traits (Figure 4). The genes GRMZM2G012224 and GRMZM2G087186 associated with PW_C, the later one also associated with PL_C, and GRMZM2G008250 with GERM_I were located inside known QTL found for both FER and fumonisin accumulation resistance.

Additionally, the gene GRMZM2G426802 related to PW_I overlapped the QTL for FSR. In a previous work, Zila et al. (2013) reported the gene GRMZM2G703598 associated with FER resistance on chromosome 1 detected in the same association population employed in this study and assayed in field conditions. This gene did not co-localize within any QTL in the map (Figure 4).

Two genes (GRMZM2G040627 and GRMZM2G025783) on chromosome 2 and one (GRMZM2G141638) on chromosome 3 related to GERM_I overlapped two QTL regions reported for FER and fumonisin accumulation (Figure 4).

The genes associated with PL_C, GRMZM2G058394 and GRMZM5G851807, were located at the ends of QTL reported for FER and fumonisin resistance on chromosome 4 (Figure 5).

On chromosome 5, two genes (GRMZM2G125432 and GRMZM2G171801) related to GERM_I were located inside QTL reported for FER and fumonisin resistance. One more gene (GRMZM2G071970) associated with the same trait was located inside a known QTL involved in FSR resistance (Figure 5). Moreover, the gene GRMZM2G111471 identified by Zila et al. (2013) was very close to the genes GRMZM2G027232 and GRMZM2G154954 for PL_I and PW_I, respectively.

One gene related to GERM_I (GRMZM2G129288) was located inside a QTL reported for fumonisin accumulation on chromosome 6 (Figure 5).

Two genes associated with GERM_I traits (GRMZM2G126010 and GRMZM2G139460) overlapped with QTL intervals for resistance to Fusarium.

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Figure 5 Localization of QTL and genes containing or adjacent to SNPs identified by GWAS on chromosomes 4, 5 and 6. Genes associated with different traits (PL_C; PL_I; PW_C; PW_I; GERM_C; GERM_I) from this study are indicated by the black arrow. Candidate genes from Zila et al. (2013) are indicated by the orange arrow. Horizontal lines of different colors represent different QTL intervals for resistance to *Fusarium* and fumonisin accumulation identified by previous reports: pink and gray (*Fusarium* ear rot and fumonisins from Lanubile et al. 2014b and Maschietto et al. 2017, respectively); green (*Fusarium* seed rot from Ju et al. 2017); blue (*Fusarium* seedling rot, plant length and weight from Septiani et al. 2019). The centromere is indicated by a red label on the chromosome.
fumonisins accumulation and FSR on chromosome 8, respectively, and one additional gene for GERM_C (GRMZM2G024799) was in the QTL for FER (Figure 6).

On chromosome 9, three genes (GRMZM2G031370, GRMZM2G058162 and GRMZM2G338376) were found associated with PL_I inside a QTL reported for fumonisins accumulation (Figure 6).

No gene associations were observed on chromosomes 7 and 10.

In the recent work by Ju et al. (2017), three QTL for FSR were found associated to three genes identified in this study, two for GERM_I and one for PW_I (Figures 4-6). This finding once again confirms how F. verticillioides infection limits germination of maize kernels and reduces seedling weight and vigor. Conversely, no correspondence was observed with QTL previously described by Septiani et al. (2013) are indicated by the orange arrow. Horizontal lines of different colors represent different QTL intervals for resistance to Fusarium and fumonisin accumulation identified by previous reports: pink and gray (Fusarium ear rot and fumonisins from Lanubile et al. 2014b and Maschietto et al. 2017, respectively); green (Fusarium seed rot from Ju et al. 2017); blue (Fusarium seedling rot, plant length and weight from Septiani et al. 2019). The centromere is indicated by a red label on the chromosome.

**Figure 6** Localization of QTL and genes containing or adjacent to SNPs identified by GWAS on chromosomes 8 and 9. Genes associated with different traits (PL_C; PL_I; PW_C; PW_I; GERM_C; GERM_I) from this study are indicated by the black arrow. Candidate genes from Zila et al. (2013) are indicated by the orange arrow. Horizontal lines of different colors represent different QTL intervals for resistance to Fusarium and fumonisin accumulation identified by previous reports: pink and gray (Fusarium ear rot and fumonisins from Lanubile et al. 2014b and Maschietto et al. 2017, respectively); green (Fusarium seed rot from Ju et al. 2017); blue (Fusarium seedling rot, plant length and weight from Septiani et al. 2019). The centromere is indicated by a red label on the chromosome.

CONCLUSIONS

In this work, a bioassay was applied to the maize association panel tested for seedling related traits to F. verticillioides resistance. GWAS analysis revealed 42 SNPs associated with three phenotypic traits: plant seedling length (PL), plant seedling weight (PW) and germination rate. In total, 25 genes were found associated with the SNPs and many of these genes had a role in disease response.

Comparison between genes found in our GWAS analysis and previous QTL mapping studies reported for resistance to FER, fumonisin accumulation and seedling traits revealed that the majority of the genes, associated with the SNPs, localized inside QTL for these traits.

These findings will help us to better understand the existing complex interaction between maize and F. verticillioides in order to improve genomic selection for Fusarium resistance at the seedling stage. Functional validation of the most recurring candidate genes will be required to verify their role in the pathways to hamper fungal infection in maize.

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