GENOME-WIDE ASSOCIATION STUDY OF RESISTANCE TO MAL DE RÍO CUARTO DISEASE IN MAIZE

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Abstract

Argentine maize has been extensively screened for incidence (INC) and severity (SEV) of Mal de Río Cuarto disease (MRC), caused by *Mal de Río Cuarto virus* (MRCV), family *Reoviridae*, genus *Fijivirus,* narrowing the breeding genetic basis. Both traits are highly heritable phenotypic measurements, and quantify the strong disease impact on grain yield. The adaptation of exotic germplasm to variation of those traits has not been explored. The aim of this work was to identify, in a non-local and diverse panel of maize inbred lines, novel genomic regions associated with resistance to MRC. First, we phenotyped 206 maize inbred lines from the International Maize and Wheat Improvement Center (CIMMYT), in several environments of the MRC-endemic area under natural virus infection, to obtain the best linear unbiased predictor (BLUP) of line effects regarding INC and SEV. A multi-environment and multi-trait mixed linear model was fitted to derive the multivariate BLUPs. Genetic variance and mean-basis heritability were high in both traits and a significant genetic correlation among them was found. Second, we performed a genome-wide association study (GWAS) by linking the BLUPs with 78,376 SNP markers available for 186 lines. The GWAS identified new alleles for resistance to MRC in six genomic regions from the exotic germoplasm. Four of them reduce symultaneously the appearance and severity of disease symptoms. Improved susceptible parental lines through marker-assisted recurrent selection would allow us to increase the resistance of maize hybrids to MRC disease.

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Introduction

Cultivated maize (*Zea mays* ssp. *mays*) was domesticated from teosinte and is one of the most widely used model plant species for fundamental research because of its wide phenotypic and genotypic diversity (Liu et al., 2020). An important viral disease of maize is Mal de Río Cuarto (MRC), which is caused by *Mal de Río Cuarto virus* (MRCV), family *Reoviridae*, genus *Fijivirus* (Milne et al., 2005). MRC is an endemic disease in Argentina and is only transmitted by plant-hoppers in a persistent propagative manner (Arneodo et al., 2002, Milne et al., 2005). *Delphacodes kuscheli* Fennah is the main natural vector of MRCV (Remes Lenicov et al., 1985). Maize plants are highly susceptible to MRC at coleoptile stage. Symptoms of infection include shortening of the internodes and the formation of galls or enations on the underside of leaves. Infected leaves sometimes undergo cross-sectional splits, with leaves being trimmed to the point that the laminae may even disappear. The inflorescences of infected plants may undergo malformations, and may even show total atrophy of both male and female flowers (Pratt and Gordon 2005). The change of the sowing date and chemical control are cultural practices used to control the plant-hopper population and therefore reduce MRCV infection. However, the most economical and environmentally friendly method for controlling this maize disease is genetic resistance (Di Renzo et al., 2004).

Resistance to MRC behaves as a quantitative trait; several molecular markers have been identified associated with resistance loci in biparental populations (linkage analysis) evaluated in the endemic area (Di Renzo et al., 2004; Rossi et al., 2015). Linkage analysis typically results in a relative low mapping resolution because of the limited number of recombination events that occur during the construction of mapping populations, whereas in genome-wide association studies (GWAS) or association mapping, historical and evolutionary recombination events can help to obtain a higher-resolution mapping (Zhu et al., 2008). The genotypic response is typically quantified using MRC severity (SEV) and incidence (INC). The meta-analysis and systematic review of QTL studies for resistance to fungi and viruses in maize performed by Rossi et al. (2019) identified several studies on viral diseases. All of them evaluated INC and SEV independently or by combining the traits in

multivariate indexes such as the disease severity index (DSI) (Chen et al., 2015; Shi et al., 2012). Even though strong environmental effects have been reported, a high phenotypic and genetic correlation was found between these traits across environments (Dintinger et al., 2005). In other plant species, a correlation between traits measuring how a disease impacts crop health was also reported (Cardoso et al., 2004) However, INC and SEV of a plant disease are independently analyzed in most genomic studies (Gowda et al., 2015; Pernet et al., 1999). Various genomic regions may carry alleles useful for reducing both INC and SEV of a viral infection in the crop. Hence, multivariate mixed models, which allow us to obtain best linear unbiased predictions (BLUPs) of genotype effects considering more than one trait and correlations among traits (Covarrubias-Pazaran 2016; Malosetti et al., 2008) could be useful to identify genomic regions with practical implications for resistance to MRC disease. Since local germplasm has being highly selected for INC and SEV of MRC in the area where the disease is endemic, the genetic basis needs to be broadened to better assess associations between the observed crop phenotypic response and the genomic information.The International Maize and Wheat Improvement Center (CIMMYT) is an important source of germplasm for the incorporation of exotic alleles into maize breeding programs worldwide. Diverse panels of maize inbred lines have been extensively genotyped and are therefore appropriate for conducting GWAS in different latitudes where they can be phenotypically evaluated. Here, a GWAS involving a highly diverse germplasm from CIMMYT, which has not been previously selected for resistance to MRC disease, was phenotypically evaluated in central Argentina. Therefore, the aim of this work was to identify new resitance alleles associated with both INC and SEV of MRC. In addition, genomic regions of resistance to MRC, which were previously identified from local biparental populations, are compared with those obtained from GWAS in the exotic maize inbred lines.

Materials and methods

Germplasm and Phenotyping

A set of 206 inbred lines, representing the three major environmental adaptations from the CIMMYT germplasm collection (Wu et al., 2016), was used for the phenotypic evaluation (Supplementary Table 1). The inbred lines were evaluated for their response to MRC disease under natural infection. The trials were performed during three crop seasons (2017-2018; 2018-2019 and 2019-2020) in Río Cuarto (64° 20′ W, 33° 8′ S) and during two crop seasons (2017-2018 and 2018-2019) in Sampacho (64º 44′ W, 33º 20′ S), two locations belonging to

an area where MRC is endemic in Argentina. An augmented partially replicated design (p-rep design) (Cullis et al., 2006) was used in all trials. P-rep design involved the use of three replicates of each of 50 test lines (so that p = 25%) and single plots of the remaining 156 lines. Each line was planted in a one-row plot of 2.5 m in length and 0.52 m between rows. In each environment, maize lines Mo17 and B73 were planted in each block as susceptible checks and BLS14 as a resistant line. The low presence of plant-hoppers in 2017-2018 resulted in poor information; therefore, the data from this crop season for both locations were removed from the GWAS. All plants of each plot were individually evaluated for symptoms at flowering stage. Symptoms were measured visually on each plant using a common ordinal scale for MRC based on the following rating system (Ornaghi et al.,1993): 0=no symptoms; 1=enation presence; 2=enation presence and shortened internodes; 3=maximal development of the disease. The individual plant scoring allowed us to quantify resistance to MRC based on the following two traits: incidence (INC), estimated as the proportion of plants presenting any one of those symptoms in each plot, and severity (SEV), which was estimated as the mean rating of all plants in the plot.

Genomic data

Molecular characterization of CIMMYT maize lines collection was performed by Wu et al. (2016). Of the 206 maize lines used in our study, a group of 186 lines were included in the genotyped collection. A dataset containing 362,008 SNP markers, publicly available on the CIMMYT Research Data Repository website (http://data.cimmyt.org/dvn), was used as genotype data of the 186 phenotyped maize lines. After minor SNP states and SNP with minor allele frequency <0.05 were removed, a total of 78,376 SNPs with low missing data rate (<35%) were kept for GWAS.

Statistical analysis

Multi-environmental phenotypic data were analyzed using the 'mmer' function of "sommer" package (Covarrubias-Pazaran, 2016) in R software (R Core Team 2016). A multi-environment and multi-trait model, as proposed by Malosetti et al. (2008), was fitted for phenotypic analysis using a dataset consisting of I genotypes, evaluated in J environments with measurements on K traits (I = 206, J = 3, K = 2). The BLUPs (West et al., 2007) of genotype effects across environments were obtained for each trait (INC and SEV). The fitted mixed linear model (MLM) (West et al., 2007) for phenotypic data included genotype effects

(considered random), trait-environment combinatorial effect (considered fixed), and genotype-environment interaction (considered random):

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

where y is the vector of phenotypic observations, and X and Z are incidence matrices. The vector β represents environmental effects regarded as fixed, u stands for random effects, and ε stands for the error terms. Random genetic effects are assumed normally distributed, N (0, σ_g^2), with the genetic (co)variance matrix (vcovG) having diagonal structure. The genotype-environment interaction (GxE) effects were assumed to be normally distributed with zero mean and compound symmetry variance-covariance structure. Errors were assumed i.i.d. N (0, σ_e^2) and independent of the other random effects.

The mean-basis heritability (H^2) (Holland et al., 2010) of INC and SEV traits was estimated using the variance components obtained from the fitted MLM, as follows:.

$$H^2 = \frac{\sigma_g^2}{\sigma_g^2 + \left(\sigma_{ge}^2/e\right) + \left(\sigma_e^2/p\right)}$$

where σ_g^2 is the genotypic variance component, σ_{ge}^2 is the genotype-environment interaction variance, σ_e^2 is the residual variance, e is the number of environments, and p is the harmonic mean of the number of plots per line across environments (Holland et al., 2010).

Genome Wide Association Study

The software TASSEL 5.2.60 (Bradbury et al., 2007) was used to perform the GWAS involving 78,376 SNPs and the BLUPs across environments for INC and SEV of MRC disease as dependent variables to avoid differences in responses due to environmental effects. The following mixed models for GWAS were fitted: 1) a general linear model (GLM) using the Q matrix obtained from the software STRUCTURE (Pritchard et al., 2000) with three groups (Wu et al., 2016) to account for the genetic correlations among lines (Q model); 2) a GLM including principal components of genomic data to model the underlying genetic structure in the population of maize lines (PCA model) (Zhao et al., 2007); 3) a mixed model including the Kinship matrix (Parisseaux and Bernardo 2004) to model genetic relationship between any two lines in the studied population (K model); 4) a mixed model including both the genetic structure suggested by STRUCTURE and the Kinship matrix (Q + K model); and

5) a mixed model including both the genetic structure suggested by PCA and the Kinship matrix (PCA + K model) (Yu et al., 2006). All fittings were compared and the most suitable model was selected by quantile-quantile plots, which compare observed -log10 (*P-value*) with the expected -log10 (*P-value*) under the null-hypothesis of no associations between marker with SEV or INC traits. A high threshold value and the procedure proposed by Li and Ji (2005) for P-value corrections were used to avoid spurious associations in multiple testing. The SNP markers were determined to be significantly associated with SEV or INC traits at a threshold of -log10 (*P-value*) > 4 (*P-value* < 0.0001). The Q-Q plots and Manhattan plots were created in "qqman" package (Turner, 2018) of software R (R Core Team 2016) using the GWAS results from TASSEL.

Results

The values of the response variable followed a nearly normal distribution for both traits, suggesting a high variability in the evaluated maize line panel (Fig. 1, Table 1). Estimates of variance component parameters from the MLM of phenotypic values revealed significant genetic variance in both traits (higher than the interaction variance). Genotypic differences explained 67.31% and 55.38% of total variability for INC and SEV of MRC disease, respectively (Table 2).

The mean-basis heritability was 0.67 for INC and 0.71 for SEV, and the genetic correlation between INC and SEV of all lines was high and statistically significant (Table 2). The correlation of genotype rankings between environments and between each environment and the data pooled across environments was high for both INC and SEV traits (Table 3).

GWAS results from the fitted models are shown as quantile–quantile plots of estimated −log10 (P-value) (Fig. 2). Based on the observed -log10 (p-value) distribution of INC and SEV, Q+K model was discarded due to its skewed distribution toward significance for both traits. The K and PCA+K models showed that some data points fell under the diagonal for both traits, which may indicate overfitting. Even though the Q and PCA models were highly competitive for SEV trait, the results from the Q model assuming a genetic population structure of three groups were better than those of the PCA model for INC; therefore, we selected the Q model as the GWAS model.

Several SNPs were statistically associated with resistance to MRC at a threshold of -log10 (P-value) > 4 (P-value < 0.0001) (Fig 2). However, we report only the genomic regions

associated with a putative candidate gene based on the B73 maize genome reference sequence (Table 4). Among the SNP markers associated with INC and SEV of MRC disease across environments, there were SNPs located very close to each other (several bp to several tens of bp). Therefore, only the SNP marker with the most statistically significant effect for a given trait was selected in each genomic region (Table 4). Under this selection criteria, a total of six genomic regions distributed in chromosomes 1, 2, 3, 6, and 8 of the maize genome were found associated with INC and/or SEV of MRC disease. Each of these genomic regions explained between 8 and 20% of the trait phenotypic variation (Table 4). Four of the six genomic regions (located in chromosomes 2, 3 and 6) were simultaneously associated with both traits. These genomic regions jointly explained 42 and 32% of phenotypic variation for INC and SEV traits, respectively. Putative candidate genes with plant defense function previously reported in such genomic regions are shown in Table 4.

Discussion

The relationship between INC and SEV is epidemiologically important for plant diseases; however, it has never been fully understood (Seem 1984). *Mal de Río Cuarto virus* is naturally transmitted in a persistent, propagative manner by an insect vector; therefore, phenotypic evaluation is performed under natural conditions, since artificial inoculation of the virus is not possible (Arneodo et al., 2002). In most of the environments selected for conducting trials, natural infestation is usually sufficient for the analysis of both INC and SEV; however, several researchers have preferred to measure INC and use quantifiable relationships to derive SEV estimation. In this work, we measured both traits and, using multi-trait models, the trait variances were adjusted by the genetic correlation between INC and SEV.

The results from the local phenotypic evaluation of INC and SEV on the CIMMYT panel showed a wide range of genotypic responses, from completely uninfected lines to completely damaged lines. The average INC of MRC disease (near 50%) of plants indicates that half of the plants of most genotypes had symptoms. The average SEV of MRC disease (close to 1) indicates that enation presence was the most frequently observed symptom in plants (Fig 1). These results are consistent with those obtained by Bonamico et al., (2012), who performed a QTL mapping of resistance to MRC using a biparental population of recombinant inbred lines; however, those authors used an index that integrates both traits rather than analyzing both traits in a multiavariate framework.

The genetic variance component showed a significant relative contribution to the total variance for both traits, INC and SEV, whereas the GxE interaction variance was relatively lower than the genetic variance. The significant genotypic variation observed in both traits reflected the high quality of the phenotypic data, which allowed us to identify genomic regions with substantive power. Therefore, the heritability across environments for both traits was higher than that reported in biparental populations (Bonamico et al., 2012; Rossi et al., 2015). A precise and reliable phenotypic evaluation is one of the main prerequisites for GWAS (Yu et al., 2008).

The genetics of resistance to MRC disease has been extensively studied in maize with biparental populations (Di Renzo et al., 2002; Rossi et al., 2015). However, the multi-environment assessment of exotic germplams has not been as much explored for the disease. In our study, a diverse panel of maize inbred lines covering different breeding programs around the world was assessed using a GWAS approach. As noted for GWAS, the presence of stratification and an unequal distribution of alleles within population subgroups can result in nonfunctional, spurious associations (Flint-Garcia et al., 2003). Consequently, different GWAS models were evaluated to control the underlying population genetic structure using the molecular information contained in 78,376 SNPs. The most suitable model for GWAS was the "Q model"; in this model, following the work of Wu et al., (2016), the Q matrix indicating genetic clustering was created assuming a population structure composed of three groups of maize lines. Those authors performed a complete molecular characterization of CIMMYT maize lines, including the lines involved in our study, and the results showed a clear separation by environmental adaptation, forming three clusters. The genetic structure in the population of lines assessed for resistance to MRC in central Argentina was also well represented by a genetic structure composed of three clusters.

Multi-trait mixed models are a suitable tool for analyzing response to INC and SEV of a plant disease and, at the same time, to control variation due to the underlying genetic correlations between traits. Previous studies on plant resistance to viral infection integrated INC and SEV traits in a severity index (DSI) for their analysis (Chen et al., 2015; Shi et al., 2012). Although the DSI contains information of both traits, the genetic basis of resistance cannot be well understood because both phenotypic measurments are combined in a unique value. In our study the genetic correlation between these traits was high. The genetic basis of this correlation can be understood in terms of pleiotropy and/or linkage (Malosetti et al., 2008). In

the multi-trait model fitted in the current study, the marker positions indicate that pleiotropy is more plausible than linkage to explain MRC INC and SEV variation among maize lines.

Genes and genomic regions conferring resistance to various pathogens often reside in clusters (McMullen & Simcox 1995; Redinbaugh et al., 2018). On bin 2.02, where a genomic region linked to both INC and SEV of MRC was mapped, three loci conferring resistance to three different viral diseases were previously identified (Dintinger et al., 2005; Luan et al., 2012; Jones et al., 2018). In adition, this cluster reported in bin 2.02 contains a major QTL resistant to Mal de Río Cuarto virus and/or Maize Rough Dwarf virus, which was patented by Martin et al. (2010). On chromosome 6, Redinbaugh et al. (2018) reported overlapping of loci conferring resistance to different viruses at 157 Mb. In a very close position, we identified a significant common genomic region for both traits and Rossi et al. (2015) reported a QTL for SEV of MRC identified in a $F_{2:3}$ population. On Chromosome 8, where the marker S8_168828138 was statistically associated with INC, Bonamico et al. (2012) and Luan et al. (2012) identified QTL for resistance to *Mal de Río Cuarto virus* and *Rice black-streaked virus*, respectively, in the same position (168 Mb).

Several genes are usually involved in plant disease resistance (Poland et al., 2009). We identified genes that may be involved in plant disease resistance; they are adjacent to the genomic regions that are significantly associated with both INC and SEV of MRC disease. A genomic region adjacent to serine-threonine protein kinase gene on chromosome 1 was identified. This family of genes is known to be involved in plant defense responses (Kump et al., 2011). Moreover, we identified genomic regions related to INC and SEV of MRC on chromosome 6 near leucine-rich repeat (LRR) domains, which have been associated with resistance genes in other diseases (Yang et al., 2017). The putative candidate genes related to genomic regions common to INC and SEV might both reduce the probability of symptom occurrence in a plant and slow down the appearance of severe symptoms, resulting in a reduction in disease incidence as well as a delay in the evolution of symptom severity.

Our results suggest that the exotic germplasm from CIMMYT has high genetic variability for resistance to MRC disease; consequently, it is a source of new alleles for breeding of the local maize germplasm. Multi-trait mixed models allow us to account for the expected genetic correlation between INC and SEV traits. Six genomic regions for resistance to MRC, four of them common to both traits, were identified using GWAS. Further research works are necessary to validate the effects of the identified candidate genes and confirm that they confer

resistance to MRC in maize across environments. The evaluation of an exotic germplasm for the response to a local endemic disease contributed to the identification of new resistance alleles, as well as to the validation of genomic regions identified in a biparental population. The multi-trait mixed model is a methodological advance that allows us to simultaneously analyze INC and SEV as disease mesurements. The genomic regions common to both traits might provide exotic alelles to local maize breeding programs, contributing to crop health. These genomic regions could be incorporated to improve susceptible parental lines through marker-assisted recurrent selection. This approach may contribute to the rapid development of maize hybrids resistant to MRC disease.

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Conflict of Interest: The authors declare that they have no conflict of interest.

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Figure 1. Frequency distribution of BLUPs of genotypic effects for incidence and severity of Mal de Rio Cuarto disease assessed across environments.

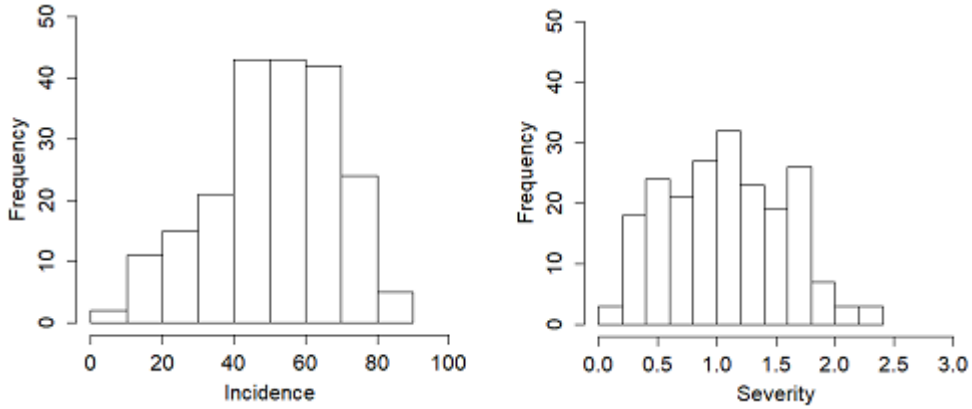

Figure 2. Manhattan plots and quantile-quantile plots of GWAS results of general linear model (Q model) for incidence (top) and severity (bottom) of Mal de Rio Cuarto disease across environments.

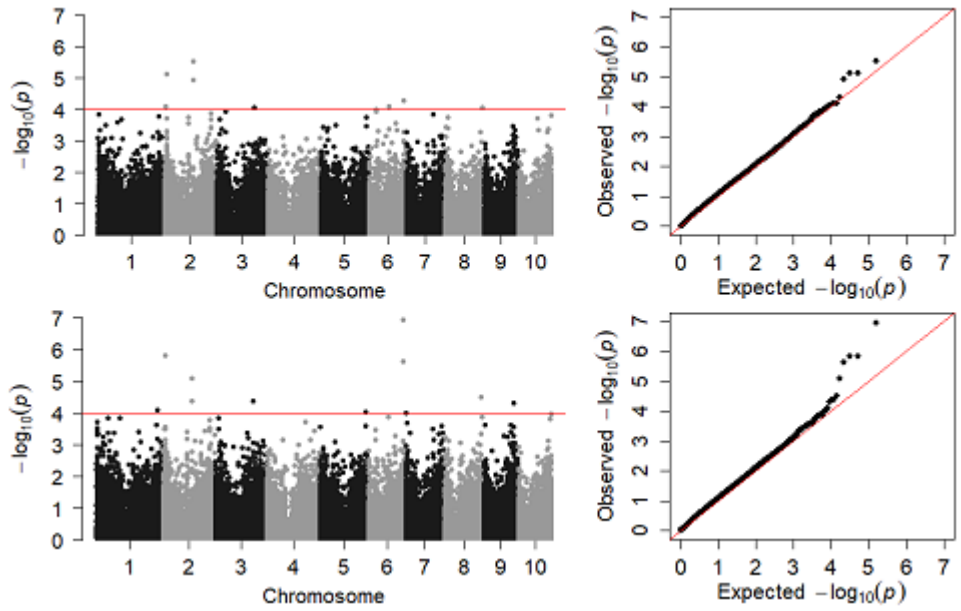

Table 1. Mean, standard deviation (SD) and coefficient of variation (CV) of 206 maize inbred lines evaluated for incidence and severity of Mal de Río Cuarto disease in a single environment and across environments.

Environments	Incidence			Severity		
	Mean	SD	CV (%)	Mean	SD	CV (%)
RC-18-19	49.72	33.67	67.72	1.04	0.88	84.03
RC-19-20	43.06	30.65	71.18	0.87	0.75	85.84
SA-18-19	66.05	35.34	53.51	1.75	1.09	62.14
Across environments	50.95	17.06	33.49	1.08	0.51	47.29

Mean values in a dimensional scale from 0 to 3 for severety and from 0 to 100% for incidence.

RC-18-19: Río Cuarto 2018-2019; SA-18-19: Sampacho 2018-2019; RC-19-20: Río Cuarto 2019-2020

Table 2. Variance components, relative variability of each component and mean-basis heritability estimates of incidence and severity in a panel of 206 maize inbred lines.

Parameters	Incidence		Severity	
	Variance	Relative variability (%)	Variance	Relative variability (%)
σ_G^2	424.40***	67.31	0.36***	55.38
σ_{GE}^2	64.01^{ns}	10.15	0.08***	12.31
σ_E^2	142.07***	22.53	0.21***	32.31
H^2	0.67		0.71	
r_g	0.93***			

σ_G^2 genetic variance component, σ_{GE}^2 genetic × environment variance component, σ_E^2 environmental variance component.

H^2 mean-basis heretability.

r_g genetic correlation.

Table 3. Correlation coefficients between environments and between each environment and the BLUPs of genetic effects of incidence and severity across environments assessed in 206 maize inbred lines.

Incidence				
Environment	RC-18-19	SA-18-19	RC-19-20	Across environments
RC-18-19	1			
SA-18-19	0.47***	1		
RC-19-20	0.44***	0.33***	1	
Across Environments	0.80***	0.80***	0.72***	1
Severity				
RC-18-19	1			
SA-18-19	0.51***	1		
RC-19-20	0.50***	0.44***	1	
Across Environments	0.82***	0.85***	0.75***	1

RC-18-19: Río Cuarto 2018-2019; SA-18-19: Sampacho 2018-2019; RC-19-20: Río Cuarto 2019-2020

*** *P value* <0.0001

Table 4. Details of identified SNP markers associated with incidence (INC) and severity (SEV) of Mal de Rio Cuarto (MRC) disease across environments.

Chromosome	Bin	Region	Marker	Alleles	Trait	p-value	R^2	-log10(p-value)	Putative candidate gene	Predicted function of candidate gene
1	1.10	1	S1_275628957	C/G	SEV	7.96×10^{-5}	0.08	4.10	Zm00001d033847	Serine/threonine-protein kinase bur1
2	2.02	2	S2_12441430	A/C	INC	7.51×10^{-6}	0.15	5.12	Zm00001d002416	putative RING zinc finger domain superfamily protein
					SEV	1.50×10^{-6}	0.17	5.82		
	2.05	3	S2_131348647	T/C	INC	2.93×10^{-6}	0.19	5.53	Zm00001d004691	Serine carboxypeptidase-
					SEV	8.01×10^{-6}	0.17	5.10		
3	3.06	4	S3_170423197	G/T	INC	8.67×10^{-5}	0.13	4.06	Zm00001d042523	Cell division cycle protein 27 homolog B
					SEV	4.29×10^{-5}	0.13	4.37		
6	6.06	5	S6_158275013	G/A	INC	5.10×10^{-5}	0.13	4.29	Zm00001d038481	putative leucine-rich repeat receptor-like protein kinase family protein
					SEV	1.11×10^{-7}	0.20	6.95		
8	8.07	6	S8_168828138	C/T	INC	8.99×10^{-5}	0.15	4.05	Zm00001d012119	Lysine-specific demethylase REF6

The exact physical position of the SNP can be inferred from the marker's name, e.g. S8_168828138: chromosome 8; 168,828,138 bp

R^2 phenotypic variance explained by the marker. A bin is an arbitrary segment of a chromosome with a pre-defined size. Each bin is designated with the chromosome number followed by a two-digit decimal.