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How to cite this manuscript

If you make reference to this version of the manuscript, use the following information:

Crespo-Herrera, L. A., Akhunov, E., Garkava-Gustavsson, L., Jordan, K. W., Smith, C. M., Singh, R. P., & Åhman, I. (2014). Mapping resistance to the bird cherry-oat aphid and the greenbug in wheat using sequence-based genotyping.

Published Version Information

Citation:

Crespo-Herrera, L. A., Akhunov, E., Garkava-Gustavsson, L., Jordan, K. W., Smith, C. M., Singh, R. P., & Åhman, I. (2014). Mapping resistance to the bird cherry-oat aphid and the greenbug in wheat using sequence-based genotyping. *Theoretical and Applied Genetics*, 127(9), 1963-1973.

Digital Object Identifier (DOI): 10.1007/s00122-014-2352-5

Publisher's Link: <http://link.springer.com/article/10.1007/s00122-014-2352-5>

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Mapping resistance to the bird cherry-oat aphid and the greenbug in wheat using sequence-based genotyping

Theoretical and Applied Genetics, 127(9), 1963-1973.

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ABSTRACT

The aphids *Rhopalosiphum padi* and *Schizaphis graminum* are important pests of common wheat (*Triticum aestivum* L.). Characterization of the genetic bases of resistance sources is crucial to facilitate the development of resistant wheat cultivars to these insects. We examined 140 recombinant inbred lines (RILs) from the cross of the susceptible wheat Seri M82 with the synthetic hexaploid wheat CWI76364, resistant to both aphid species. The RILs were phenotyped for *R. padi* antibiosis and tolerance traits. Phenotyping of *S. graminum* resistance was based on leaf chlorosis in a greenhouse screening, and also on the number of *S. graminum* per tiller in a field trial. Seedling pubescence was scored in each RIL. Using a sequence-based genotyping method we located genomic regions associated to these resistance traits. One QTL for *R. padi* antibiosis was found in chromosome 4BL; it explained 10.2% of phenotypic variation and was located 14.6 cM apart from the pubescence locus. However, we did not find any association between plant pubescence and the other resistance traits. We found two QTLs for tolerance to *R. padi* in chromosomes 5AL and 5BL, with an epistatic interaction between a locus in chromosome 3AL and the tolerance QTL in 5AL. These genomic regions together explained about 35% of the phenotypic variation. We confirmed the location of a previously reported gene for *S. graminum* resistance (*Gba*) in 7DL and found an additional, novel QTL associated with the number of aphids per tiller in chromosome 2DL. This is the first report where resistance to *R. padi* in wheat is mapped and also where chromosome 2DL shown to be associated with *S. graminum* resistance.

Key words: *Rhopalosiphum padi*, *Schizaphis graminum*, synthetic hexaploid wheat, genotyping by sequencing, resistance, QTLs.

INTRODUCTION

The aphids *Rhopalosiphum padi* L. and *Schizaphis graminum* (Rondani) are two of the most serious pests of wheat (*Triticum aestivum* L.), and can significantly reduce wheat yields by 30-40% solely due to feeding and up to 60% when such damage is combined with virus infection (Kieckhefer and Gellner 1992; Voss et al. 1997; Riedell et al. 2003). Both aphid species are widely distributed in regions where wheat is grown (Blackman and Eastop 2007), however, *S. graminum* is not a wheat pest in northern Europe. One way of contributing to reduce the damage caused by these pests is by breeding resistant varieties.

Including resistance to these aphids as an additional component in conventional wheat breeding programs is challenging. Phenotypic selection for aphid resistance is difficult to apply without sacrificing other breeding goals. It

requires considerable logistic efforts to maintain large segregating populations in various generations during selection under homogenous aphid pressures across time and space. However, the identification of molecular markers associated with aphid resistance/tolerance genes can greatly facilitate the addition of this component into wheat breeding programs. Therefore, it is crucial to characterize and understand the genetic bases of aphid resistance in wheat germplasm.

Resistance to insects is generally classified as antibiosis, antixenosis and tolerance (Smith 2005). Antibiosis builds on plant characteristics that negatively affect insect physiology; causing higher mortality rates, longer development periods, reduced weight gain, etc. Antixenosis is the reduced suitability of a plant to serve as a host for feeding and reproduction via plant traits that influence the insect host-selection behavior. Tolerance is the ability of a plant to withstand or recover from insect damage without compromising insect behavior or physiology. All three components of insect resistance tend to be present in resistant plants to a certain level. However, oftentimes there is one component that predominates over the others making a plant genotype more antibiotic, antixenotic or tolerant. Some complications at the categorization of insect resistance are, 1) that antixenosis expressed as lower feeding rate may reduce insect performance and then be interpreted as antibiosis and 2) that tolerance measured as reduced insect-specific plant symptoms may be due to fewer or less of insect feeding and not due to a plant response. In the present study, we measured weight gain of *R. padi* nymphs after a certain feeding period to estimate antibiosis and reduced seedling biomass under *R. padi* feeding pressure to estimate tolerance. Furthermore, we scored leaf chlorosis as an estimate of plant tolerance to *S. graminum* and measured *S. graminum* density in a field trial to estimate antixenosis/antibiosis.

The wild relatives of wheat are rich sources of genetic variation that may confer resistance to various biotic stresses. One way to incorporate such variation in bread wheat is by synthesizing hexaploid wheats (SHWs) from the cross of a tetraploid species with *Aegilops tauschii* Coss. Several resistance traits to various pests and diseases have been reported in SHWs, and in some cases they have been successfully deployed into commercial varieties (Ogbonnaya et al. 2013). For instance in the case of aphids, Smith and Starkey (2003), evaluated a large set of SHWs for resistance to *S. graminum* and found high levels of variation that was further characterized by Zhu et al. (2005).

Efforts to deploy resistance to *S. graminum* in wheat were made since the 1950's (Porter et al. 1997; Berzonsky et al. 2003) and this has led to the identification of several resistance genes. Currently there are 14 major genes for resistance to *S. graminum* in wheat that have been identified (McIntosh et al. 2010; Crespo-Herrera 2012). Of those, one is from *Triticum turgidum* L., two from *Secale cereale* L., one from *T. aestivum*, one from *Aegilops speltoides* Tausch, and 9 from *A. tauschii*. Some of these genes have been incorporated into wheat cultivars, for instance the gene *Gb3* from *A. tauschii* (Ogbonnaya et al. 2013). There are several *S. graminum* biotypes that differ in their virulence patterns to these resistance genes; biotypes E and I are the most common on wheat in the USA (Burd and Porter 2006).

Despite that *R. padi* is considered to be an important pest in wheat, there are no resistant cultivars developed for this aphid species. This may be because *R. padi* damage is not as evident as for other aphid species; for instance *S. graminum*, which does cause clear plant symptoms and so the selection of resistant-plant progeny can be based on that trait. Additionally, the polyphagy and wide host adaptation of *R. padi* can make it difficult to find resistance sources with adequate protection levels. Possibly this is why there are no published studies on the genetics of *R. padi* resistance in wheat, and so far no aphid biotypes of this species have been reported either. However, resistance to this species has been mapped in barley, and a quantitative trait locus (QTL) in chromosome 3H that explained 9% of the aphid growth variation was reported by Cheung et al. (2010).

Next generation sequencing technologies provide great opportunities to unravel the genetic bases of quantitative traits by supplying large amounts of data in a cost and time effective manner. Genotyping by sequencing (GBS) is one of the applications. In the development of GBS libraries the genome complexity is reduced by the use of methylation-sensitive restriction enzymes and in combination with multiplex sequencing it is possible to genotype

entire populations efficiently (Elshire et al. 2011; Poland et al. 2012). This is particularly useful for species with large genomes such as wheat, where sequencing needs to target non-duplicated regions of the genome in order to produce high quality maps (Spindel et al. 2013). GBS methods can be applied in bi-parental populations to locate genomic regions associated to various agronomic traits of interest (Saintenac et al. 2013).

In this study we identified genomic regions associated to various traits related to resistance against *R. padi* and *S. graminum* in a SHW by using sequencing-based genotyping methods. This is the first time that antibiosis and tolerance to *R. padi* was mapped in wheat and also that resistance to *S. graminum* was found in chromosome 2DL.

MATERIALS AND METHODS

Plant material

We developed a mapping population of 140 F6 recombinant inbred lines (RILs) with the single-head descent method at the International Maize and Wheat Improvement Center (CIMMYT) in Mexico. The progenitors of the population were the spring wheat Seri M82 (aphid susceptible) and the SHW CWI76364 (aphid resistant). The pedigree of CWI76364 is: *Triticum dicoccum* PI 94623/*A. tauschii* (1027) (Lage et al. 2003). This *A. tauschii* accession was also progenitor of the population where the *Gba* resistance gene was mapped by Zhu et al. (2005).

Phenotyping and statistical analyses of phenotypic data

The 140 RILs were phenotyped for resistance to *R. padi* and *S. graminum*. Evaluations of *R. padi* resistance were conducted in the Department of Plant Breeding at the Swedish University of Agricultural Sciences (SLU). *S. graminum* resistance evaluations were performed in the Department of Entomology at the Kansas State University (KSU) and also at CIMMYT's breeding station in Ciudad Obregon, Mexico.

All statistical analyses of the phenotypic data were made with SAS statistical software v9.3 (SAS Institute Inc 2011).

Rhopalosiphum padi phenotyping

Antibiosis and tolerance were evaluated with virus-free individuals of *R. padi* reared on oat plants under greenhouse conditions at ca. 22°C, with a minimum of 16h of light, supplemented when needed with 400W high-pressure sodium lamps.

Antibiosis evaluations started by placing eight seeds of each RIL on moist filter paper in Petri dishes. Dishes were kept in a refrigerator at 5°C during 3 days and thereafter for 2 days at room temperature. After that period four germinated seeds were singly transplanted in 10 cm diameter plastic pots (300 ml) filled with Weibull's Kronmull® potting soil with Leca. Seedlings were grown in a walk-in climate controlled chamber at 22°C, 80% RH and 16h light at the intensity of 250 μmol photons m⁻²s⁻¹ at plant level. The RILs were tested in seven time-separated incomplete blocks with a maximum of 24 lines per incomplete block including both parents. Each line was replicated four times within each incomplete block.

When plants reached 2-3 leaf stage they were individually exposed to five 1st instar nymphs that were born within a period of 24 hours. These new-born nymphs were obtained from alate individuals previously caged on oat plants for 24 hours. The new-born nymphs were confined at the plant base with transparent cylindrical acrylic cages (2 cm diameter, 5 cm length), sealed with cotton wool at the bottom and the top. Four days after infestation aphids were individually weighted on a microbalance (Mettler M3). Analysis of variance and least-square means were estimated using the MIXED procedure in SAS, with RILs and replications nested within incomplete blocks. Variance

components were estimated to calculate heritability. For the QTL analysis, the mean aphid weight on each RIL was expressed as the proportion of the mean aphid weight displayed for the susceptible parent.

Tolerance of each RIL was measured as biomass reduction in an augmented split plot designed experiment with two treatments, Infested (1) and Non-infested (2). Each parent of the population was repeated 15 times. Biomass reduction was calculated with the formula: $1 - (I/NI)$, where I is equal to the plant biomass of the aphid-infested RIL, and NI is equal to the plant biomass of the same non-infested RIL. The experiment was conducted under the same greenhouse conditions as for *R. padi* rearing, using the same type of soil as in the antibiosis tests. First, four seeds of each RIL were sown in flats (41 cm x 61 cm x 11 cm). Three days after germination, excess plants were gently pulled out to allow only one plant to grow, and to select plants of about the same size for the application of treatments. Seedlings of each treatment were grown in separate flats respectively. Plants of treatment 1 were infested every second day with aphids at an approximate density of 45 aphids/plant. Infestations started when seedlings were at 2-3 leaf stage. Plants of treatment 2 were treated with the systemic insecticide Confidor WG® (Bayer CropScience) at a dose of 3.5 % of active ingredient by pouring the solution in the soil (Dunn et al. 2007). Fifteen days later, plants of both treatments were cut at the soil level and dried during 72 hours at 70°C. Plants were then weighted on an analytical balance (Sartorius ME215P). Analysis of variance was performed with the MIXED procedure of SAS. Parents and treatments were fixed effects, whereas the RILs and their interaction with the treatments were treated as random effects in the model. Variance components were calculated to estimate heritability.

Schizaphis graminum phenotyping

Evaluations of *S. graminum* resistance consisted of two separate tests, one testing for leaf symptoms and another for population build up in the field. Screening for leaf chlorosis was performed under greenhouse conditions at ca. 22°C, 80% RH and 16 h light at KSU. Virus-free starter colonies of *S. graminum* biotype E were obtained from USDA-ARS and maintained on Jagger wheat. Biotype E is common and virulent to wheat carrying resistance genes *Gb1* and *Gb2* (Burd and Porter 2006) but not to the other 12 known resistance genes (Crespo-Herrera 2012). Resistance was scored as R (resistant) or S (susceptible) based on leaf chlorosis symptoms in the same fashion as by Zhu et al. (2005). The entries of the population were sown in hill plots of 6-8 seeds/hill in a complete randomized block design with four replicates. Plants were infested with an approximate density of 5 aphids/hill plot at 2-3 leaf stage. Resistance was scored when the susceptible parent showed more than 50% chlorosis. Statistical analysis of R/S responses was made with the LOGISTIC procedure of SAS.

Outbreaks of *S. graminum* commonly occur in Northwestern Mexico during the wheat growing season where we performed the field evaluation. The field test was conducted at CIMMYT's Ciudad Obregon experimental station, in Mexico (27° 37' N, 109° 93' W) during the winter season 2012–2013. The 140 RILs were sown on December 20th, 2012. The experiment was laid out in a rectangular row-column augmented design (6x30), with the population's parents replicated 12 times. Eight to ten seeds of each RIL were planted in a 10 cm hill plot in a bed-planting system with 0.8 m of distance between beds and 1m distance between hill plots along the rows. The trial was irrigated six times throughout the crop season by surface irrigation. Fertilization rate was 200–50 (N–P), of which 50–50 was applied at sowing, and 150–00 was applied 3–4 weeks after sowing along with the first irrigation. Weeds were controlled manually. The number of aphids in ten tillers was counted in each hill plot when *S. graminum* reached its highest population density in a neighboring trial. The number of aphids/tiller was transformed to a logarithmic scale before the statistical analysis. The MIXED procedure of SAS was used to make the analysis of variance. Parents were fixed effects in the model, whereas the RILs were treated as random effects. Variance components were used for the estimation of heritability.

Pubescence phenotyping

Seedlings of the mapping population were grown in a greenhouse at CIMMYT's headquarters (19° 32 ' N, 98° 50' W) in October 2012 at an average temperature of 25°C and natural day-length conditions. Leaf pubescence was scored as either present or absent when the plants had reached 2-3 leaf stage.

Genotyping

Seedlings of the 140 RILs and the parents were grown in a greenhouse in the Department of Plant Pathology at the Kansas State University. Plant tissue was collected when plants reached 2-4 leaf stage. Extractions of DNA were made with a QUIAGEN DNeasy 96 Plant Kit[®], by following the manufacturer instructions. DNA was quantified using the Quanti-iT[™] PicoGreen[®] and concentrations were then normalized to 20 ng/μl.

Genotyping by sequencing (GBS) libraries were obtained following the protocol reported by Saitenac et al. (2013). The combination of PstI (barcoded adapter) and MseI (Y-adapter) restriction enzymes was used. We used a set of 96 barcodes with sticky ends complementary to the 3' overhang of PstI and MseI-Y-adapters (Saitenac et al. 2013). Prior to the use of the adapters, the common primer (5'-CTCGGCATTCTGCTGAACCGCTCTTCCGATCT-3') was annealed with the enzyme specific primer of MseI (5'-taAGATCGGAAGAGCGGGGACTTTAAGC-3') to prepare the Y-adapters. Annealing was done with a thermal cycler in 10 nM Tris-HCl and 50 nM NaCl buffer. Temperature was reduced from 95°C to 30°C in 65 cycles at a rate of 1°C/min. Barcode adapters were then adjusted to a concentration of 0.2μM.

DNA samples were pipetted in two 96-well plates of 72 and 70 samples each. Restriction, ligation and amplification processes were performed to construct the genomic libraries (Poland et al. 2012; Saitenac et al. 2013). Restriction of DNA was made with a restriction mix that consisted of 20μl of each sample, 3.0μl of NEB T4 DNA ligase buffer 3, 0.5μl of PstI (10 units), 1 μl of MseI (10 units) and 5.5μl of H₂O. Ligation was performed by adding to the restriction digest (30μl) of each sample, 3μl of the unique barcode adapter, 8μl of the Y-adapter and 9μl of ligation NEB Master Mix (2 μl of T4 DNA ligase buffer, 0.5μl T4 DNA ligase and 6.5μl water). Samples were then incubated at 22°C for two hours and maintained at 65°C for 20 minutes to inactivate the T4 DNA ligase. Before the amplification step, the samples were purified with a QUIAGEN QIAquick PCR Purification Kit[®]. Then 5μl of each sample were pooled in one tube for each 96-well plate and eluted in 50μl volume. Samples were amplified with 10μM of PCR primers 5'-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT-3' (forward) and 5'-CAAGCAGAAGACGGCATAACGAGATCGGTCTCGGCATTCTGCTGAA-3' (reverse) using a Taq 2x Master Mix from NEB. There were 18 PCR cycles of 95°C (30s), 62°C (10s), 68°C (20s) that were terminated with a final extension at 72°C for 5 minutes. The size and distribution of the DNA fragments in the genomic libraries were analyzed with the Bioanalyzer Aligen DNA 1000. Libraries were sequenced in an Illumina HiSeq2000 flow-cell machine.

SNP calling and QTL analysis.

We processed the GBS reads for SNP calling with the Universal Network Enabled Analysis Kit (UNEAK) implemented in TASSEL 3.0 standalone version (Lu et al. 2013). The GBS raw data from the Illumina HiSeq2000 machine were first trimmed to a length of 64 bp to keep high quality sequences, then identical reads were grouped into tags; further these tags were pairwise aligned to identify single base pair mismatches, which represented candidate SNPs. Each pairwise alignment represents a node in a network; the complex networks and the ones without a single reciprocal mismatch were discarded with a network filter at an error rate tolerance of 0.03 to end up only with the reciprocal tag pairs that were used for SNP calling. After processing the Illumina Fastq files with the UNEAK pipeline, a total of 1313 GBS markers were used for the QTL analysis.

We made the linkage groups with the 1313 GBS markers, and also with the categorical responses of each RIL to *S. graminum* R/S leaf symptoms and pubescence present/absent scores. We used the software ICIMapping (Li et al. 2007) with a LOD score of 9 for grouping the markers, the Traveling Salesman algorithm for ordering, and a

window size of 5 cM for rippling the linkage groups. Linkage groups having less than three markers or markers with no linkage were discarded from the analysis (altogether three out of 25). For assigning chromosome groups to the genomic regions of interests, 20% of the marker sequences in each LG were systematically searched with the Basic Local Alignment Search Tool (BLAST) in the wheat genome sequence published by Brenchley et al. (2012) and available at EnsemblPlants (http://plants.ensembl.org/Triticum_aestivum/Info/Index).

The program ICIMapping was also used to perform inclusive-composite interval QTL mapping. Significance threshold for the 10% tail of null distribution was obtained with a run of 1000 permutations and a 0.001 probability value for markers to enter into the QTL model (Da Costa E Silva et al. 2012a; Da Costa E Silva et al. 2012b). Linkage groups and QTLs were plotted with MapChart software (Voorrips 2002).

Additionally, to determine if QTLs associated to *R. padi* tolerance were related to plant growth *per se*, we conducted a multi-environmental QTL analysis with ICIMapping, where treatments Infested and Non-infested were considered as different environments.

To identify pairs of interacting loci we made a 2-dimensional 2-QTL scan with the R/qtl package in the R software v3.0.1 (Broman et al. 2003; R core team 2013). The conditional genotype probabilities of the markers were first calculated with the “calc.genoprob” instruction. The LOD score of the model including an epistatic term and the LOD score of the model accounting only for additive effects were obtained with the “scantwo” instruction. A run of 1000 permutations was made to set the threshold of the 5% tail of the null distribution. If interactions between loci were identified with the two-dimensional scan, then the positions of the main QTLs and the epistatic loci were used to estimate the effect of interactions in a multiple interval mapping framework in the R/qtl package.

A further analysis of interacting markers was made by using the MIXED procedure in SAS. We calculated least-square means for the phenotypes of the groups of genotype combinations given by the markers linked to the genomic regions of interest, and used Tukey’s test for comparisons of means to assess the difference among phenotype values given by the marker genotypes.

RESULTS

There were 22 linkage groups (LGs) used for the analysis, spanning a total length of 2252.7 cM based on 1309 GBS markers. Thus, there was on average one marker every 1.72 cM. The BLAST analysis of the GBS sequences showed that all 21 wheat chromosomes were represented by the LGs, based on identity values higher than 80%. The remaining LG was assigned to the chromosome arm 4DL.

Antibiosis to *R. padi* and plant pubescence

The phenotypic data for antibiosis did not diverge from a normal distribution according to the Anderson-Darling test ($A^2 = 0.33$; $p > 0.25$) but it appeared to be left-tailed ($k_3 = -0.1$) (Figure 1). In chromosome 4BL we found one genomic region significantly associated to *R. padi* antibiosis. This region was flanked by the markers TP48882 and TP31989 within an interval of 4.9 cM (Figure 2), and explained 10.2% of the total phenotypic variation (Table 1). Hereafter this locus will be referred to as *Rp1*. The closest marker TP48882 was 0.1 cM away from *Rp1*. The heritability was 15.7% (Table 1).

The genomic region associated to pubescence was also located in chromosome 4BL, at a distance of 14.6 cM from the antibiosis QTL. The pubescence screening showed 41.4% of the population to be pubescent and 58.6% glabrous, fitting a 1:1 segregation ratio ($df = 1$; $X^2 = 3.55$; $p = 0.06$), and indicating single gene inheritance. Groupings of RILs based on presence/absence of leaf pubescence showed no effect on *R. padi* antibiosis ($df = 1, 135$; $F = 0.323$; $p =$

0.574) or any other trait measured in this study. There were no epistatic interactions found in the two-dimensional scan.

Tolerance to *R. padi*

The phenotypic data for *R. padi* tolerance did not diverge from a normal distribution according to the Anderson-Darling test ($A^2 = 0.45$; $p > 0.25$) but appeared to be right-tailed ($k_3 = 0.33$) (Figure 1). Two significant QTLs associated to *R. padi* tolerance were found, in chromosomes 5AL and 5BL, explaining 14.5 and 5.7 % of the phenotypic variation, respectively (Table 2). The closest markers to these genomic regions are TP3728 and TP3351, respectively (Figure 2). Hereafter these loci will be referred to as *Rp2* and *Rp3*, respectively. The heritability for this trait was 36.5%.

The two-dimensional scan revealed one epistatic locus present in chromosome 3AL interacting with the marker TP3728 associated to *Rp2* (Figure 3). The epistatic locus is closest to the marker TP5978, and herein referred as *EnRp2*. The interaction accounted for 5.0 % of the phenotypic variation (Table 2). The percentage of the total phenotypic variation explained by the QTL model was 35.1%.

The analysis of variance based on the marker classes showed that the interaction TP3728 * TP59798 was significant ($df = 1, 81$; $F = 16.26$; $p = 0.0001$; Figure 4a), while there was no effect of TP59798 marker alone ($df = 1, 81$; $F = 1.23$; $p = 0.2721$). Figure 4b shows the additive effect of the two loci *Rp2* and *Rp3*. The comparison of means for all the combinations of these marker genotypes showed that the level of biomass reduction is lowest when *Rp2* (TP3728) from the resistant parent is combined with the allele from the susceptible parent of marker TP59798 (Figure 5). The comparison of means also showed that TP59798 has no effect on *R. padi* tolerance (Figure 5).

The multi-environmental QTL analysis did not show genomic regions for plant growth *per se* to be associated to *R. padi* tolerance, and therefore, this data is not shown.

Resistance to *S. graminum*

The phenotypic data for number of *S. graminum*/tiller in the field did not diverge from a normal distribution according to the Anderson-Darling test ($A^2 = 0.51$; $p = 0.193$) but showed to be left-tailed ($k_3 = -0.3$) (Figure 1). Greenhouse phenotyping for *S. graminum*-induced chlorosis showed that 55.7% of the RILs were resistant and 44.3% were susceptible and this did not deviate from the expected segregation ratio of 1:1 ($df = 1$; $X^2 = 1.82$; $p = 0.17$) for a single locus, most likely the previously mapped gene *Gba* by Zhu et al. (2005). The linkage analysis placed this locus in chromosome 7DL between markers TP81905 and TP13131 (Figure 2). Additionally, *Gba* was significantly associated to the number of *S. graminum*/tiller in the QTL analysis, explaining 16.6% of the phenotypic variation (Table 1).

Another genomic region, in chromosome 2DL, was also associated to the number of *S. graminum*/tiller, explaining 10.2% of the phenotypic variation (Table 1, Figure 2). The QTL model for aphids/tiller explained 29.1% of the total phenotypic variation, whereas the heritability was 57%. The two-dimensional scan did not show any significant interaction between pairs of loci.

DISCUSSION

The next generation sequencing technologies provide large amounts of information and are suitable for identifying novel genomic regions associated to plant stresses, evolutionary studies and genome sequencing research. GBS in particular has proven to be very adequate for these tasks (Poland et al. 2012; Saintenac et al. 2013; Lu et al. 2013). Here we constructed GBS libraries and employed QTL analyses in an F6 RIL population to map known and novel genomic regions associated to resistance to two aphid species in wheat.

We found one genomic region associated to *R. padi* antibiosis in chromosome 4BL (*Rp1*), and a plant pubescence locus in the same chromosome arm. However, they were separated by a distance of 14.6 cM, and classification of RILs based on pubescence data showed no effect on antibiosis to *R. padi*. Some studies (Roberts and Foster 1983; Webster et al. 1994), but not all (Webster 1990; Papp and Mesterhazy 1993), have suggested that one possible cause for aphid resistance in wheat is the presence of trichomes in leaves. However, in our study neither *R. padi* nor *S. graminum* resistance was related to such a plant trait. This indicates that *R. padi* antibiosis and the number of *S. graminum*/tiller are caused by mechanisms different from pubescence in the resistant genotype CWI76364. So far, there are no other published reports that have investigated the genetic association between aphid resistance and plant pubescence in wheat. However, not only presence or absence of pubescence but also density and structure of the pubescence can be of importance for the resistance effect, as it has been shown in other plant species and insect herbivores, mainly chewers and miners (Dalin et al. 2008). We did not measure such characteristics in the pubescent genotypes since we found no differences in aphid resistance between glabrous and pubescent individuals.

It is probable that the pubescence locus we found is allelic to, or the same as the hairy leaf gene *Hll* previously reported by Dobrovolskaya et al. (2007) in chromosome 4BL of *Triticum dicoccoides* L. However further evaluations are required to fully determine if this locus is the same as the one we found in *Triticum dicoccum* Schrank.

The genes *Tbx1* and *Tbx2* are other sets of genes previously discussed as resistance-related and found located in the proximal section of chromosome 4BL. These genes are involved in the synthesis of hydroxamic acids (hx) (Nomura et al. 2003). Those compounds have been reported to play an important role in the defense of gramineous plants when they are attacked by herbivores, causing antibiotic effects on insects including various aphid species (Frey et al. 1997; Niemeyer 2009). There are five hx-related genes in wheat that are identified as *TaBx1* through *TaBx5* (Nomura et al. 2002; Nomura et al. 2003). These genes are located in chromosomes 4 and 5 of the three wheat genomes, and are involved in catalyzing the hydroxylation and ring expansion of indole-3-glycerol phosphate to synthesize DIBOA and DIMBOA (Frey et al. 1997; Nomura et al. 2003). Both DIBOA and DIMBOA are present in hexaploid wheat, however, only the latter appears to be present in high concentrations (Niemeyer et al. 1992). Niemeyer et al. (1992) found that the ancestor of A and D genomes of hexaploid wheat possesses low concentrations of hx, whereas *Aegilops speltoides* Tausch, the most likely donor of the B genome, and tetraploid wheat (AABB) possess high concentrations of DIMBOA, suggesting that the B genome is responsible for the main part of the synthesis of hx in hexaploid wheat. From our results is not possible determine whether *Rp1* is associated or not to *Tbx* genes, but it would be of great interest to analyze DIMBOA concentrations in the population.

Tolerance is an attractive component of insect resistance, as this trait does not pose any selection pressure on the insects and hence the risk of developing new virulent strains is absent compared to a scenario where insects are under constant selection due to antixenosis or antibiosis. In terms of crop management, tolerance may facilitate other aphid control methods, since when a control action is taken in commercial farms, aphids might already have built up their population levels above the economic threshold on a susceptible cultivar. A tolerant cultivar may endure higher aphid populations so there is more time for localizing the infestation and if necessary apply insecticides in the field.

Tolerant and susceptible plants differ in the quantity of biomass they produce under insect stress (Smith 2005). Causes for this can be due to differences in photosynthetic rate, allocation patterns and nutrient uptake (Rosenthal and Kotanen 1994). Since tolerance is related to various fundamental physiological mechanisms in plants, which are all influenced both by intrinsic and extrinsic factors, tolerance is a very complex and difficult trait to measure (Rosenthal and Kotanen 1994). Besides, it is often present along with antibiosis and antixenosis and it is also difficult to separate the tolerance effect from the others. Still, we were able to find genomic regions associated to wheat seedling tolerance to *R. padi* in chromosomes 5AL and 5BL (*Rp2* and *Rp3*, respectively). Since we measured tolerance to *R. padi* as biomass reduction, we considered the possibility that the accumulation of plant biomass *per se* may have contributed to tolerance, as plants with higher growth rates might have less biomass reduction due to aphid feeding. Therefore we conducted a multi-environmental QTL analysis to identify genomic regions associated to plant

biomass *per se*, but we found that *Rp2* and *Rp3* were unrelated to it. This indicates that the regions we identified are only related to biomass reduction under *R. padi* stress and their expression might be induced by aphid feeding.

Since tolerance is a complex trait that involves several plant processes, its genetic effect can partly be explained by epistatic interactions. The implementation of the two-dimensional scan as described by Broman (2003), evaluates all pairwise interactions across the genome by comparing the log₁₀ likelihood ratio tests of two-QTL models with and without the epistatic term, as well as with the single-QTL models to finally obtain the LOD scores of such comparisons. The two-dimensional scan of *R. padi* tolerance displayed a significant epistatic interaction that changes the magnitude of *Rp2* caused by a genomic region located in 3AL, linked to the marker TP59798 (*EnRp2*). Tolerance to *R. padi* appears to be enhanced when the marker allele of TP59798 from the susceptible parent Seri M82 is present along with *Rp2*. The epistatic locus *EnRp2* had in itself no significant effect on biomass reduction due to *R. padi* feeding. According to Mackay (2013) the conditions for identification of epistatic interaction are optimal in biparental mapping populations because allele frequencies tend to be equal to 0.5 and thus the epistatic variance is maximized. On the other hand, the effect of the interactions can be significant even though the interacting loci have no significant effect individually (Mackay 2013).

Evaluation of tolerance to *R. padi* is difficult since the absence of visual symptoms requires the measurement of plant growth under aphid-infested and non-infested treatments. Consequently the phenotyping entails stringent conditions, such as to guarantee that each plant genotype in the pair of treatments have approximately the same starting size, something which can be highly influenced by seed quality and growing conditions. Additionally, to rule out antibiosis and/or antixenosis effects is important to ensure that plants have approximately the same aphid density over time. To meet these requirements in the evaluations we selected pairs of seedlings with approximately the same size and periodically infested with aphids to keep approximately the same aphid density throughout the phenotyping process. However, once proven to be of significant importance for biomass and eventually seed production, molecular markers such as those provided by our study allow marker-based selection in wheat breeding material without all the efforts needed to phenotype segregating populations and careful control of test conditions for phenotyping as described above.

There are 14 previously reported *S. graminum* resistance genes in wheat and wheat relatives, and nine of those have been found in the chromosome 7DL from *A. tauschii* (McIntosh et al. 2010). However, six of the nine genes reported in 7DL may be allelic or closely linked to *Gb3* (Zhu et al. 2005). Among those, the *Gba* gene originates from the same *A. tauschii* (1027) accession used in the development of CWI76364. In the present study we were able to confirm this resistance locus in chromosome 7DL by sequenced-based genotyping.

In a microarray study comparing plants with and without *S. graminum*, Reddy et al. (2013) showed that aphid-infested plants carrying *Gb3* gene prevent cell wall modification and consequently cell death, and also down-regulate genes for the synthesis of secondary metabolites related to plant defenses. Most probably the tolerance component of *Gba* plants is explained by similar molecular mechanisms. Resistance characterization of the *Gb3* gene has shown that it also confers both antixenotic and antibiotic effect on biotype E of *S. graminum* (Weng et al. 2004). This may explain the lower number of aphids/tiller that we found in the field, in our case caused by *Gba*. This makes the *Gba* gene a good candidate to be deployed in elite wheat germplasm.

Additionally, we found chromosome 2DL to be a novel region associated to the frequency of *S. graminum*/tiller in the field. This QTL, here referred to as *QGb8*, contributed 10.2% of the phenotypic variation. We attribute that the 2DL region was not previously reported in *A. tauschii* (1027) because it was associated to aphids/tiller in the field, whereas Zhu et al. (2005) evaluated symptoms based on chlorosis (tolerance) in a greenhouse test similar to ours, where we also did not find any association to 2DL. It is possible that *QGb8* has a predominantly antixenotic effect on *S. graminum* since Lage et al. (Lage et al. 2003) showed that *A. tauschii* accession#1027 was the most antixenotic among 12 studied SHWs. However, reduced aphids/tiller could also be due to antibiosis since CWI76364 showed to be more antixenotic, antibiotic and tolerant than the susceptible cultivar Seri M82 (Lage et al. 2003). Further

investigation is required to confirm and characterize *QGb8* and determine the type of resistance it confers against *S. graminum*.

Another possible explanation for the new QTL for *S. graminum* resistance might be that there was an uncharacterized biotype of this aphid in our field. We did not try to determine the biotypes of *S. graminum*, however Burd and Porter (2006) reported that biotype E and I are the most common in wheat in USA.

All phenotypic responses fitted a normal distribution with some degree of skewness, possibly because the environmental variance was relatively large compared to the genetic variance (Lynch and Walsh 1998). This is particularly the case for *R. padi* antibiosis where we observed a low heritability estimate. However, low heritability seems to be a common feature of insect life history traits, since they are highly influenced by the environment (Price and Schluter 1991).

In conclusion, we were able to utilize a sequence-based genotyping method to determine the genetic bases of resistance to *R. padi* and *S. graminum* in the synthetic hexaploid wheat CWI76364. Derived from this, we found one locus for *R. padi* antibiosis (*Rp1*) in chromosome 4BL and two loci for *R. padi* tolerance (*Rp2* and *Rp3*) in chromosomes 5AL and 5BL, respectively. We also found one locus that originates from the susceptible parent (*EnRp2*) that enhances the effect of *Rp2*. From these results we can also conclude that resistance to *R. padi* originates from *T. dicoccum* in the CWI76364/Seri M82 population. This is the first report on the genetic mapping of antibiosis, tolerance and epistatic effects against *R. padi* in hexaploid wheat. Using GBS, we were able to re-map the *Gba S. graminum* resistance gene, and were also able to find in chromosome 2DL a genomic region (*QGb8*) associated to the number of *S. graminum*/tiller in the field. Plant pubescence was not related to any of the resistance traits we measured for any of the aphid species. The identification of the GBS markers associated with the resistance loci will help to fine map those genomic regions and to develop new molecular markers that are easier to apply in wheat breeding. We think that the loci we found are promising sources to be deployed in elite wheat germplasm, since several resistance mechanisms should make the resistance more durable. Nonetheless, more efforts in finding and characterizing additional sources of resistance are needed in order to further enhance wheat cultivars' resistance to *S. graminum* and *R. padi*. By increasing the knowledge in this area it will be possible to diminish the use of pesticides and consequently it will contribute to a more environment friendly production of wheat.

Acknowledgments

We thank the Monsanto's Beachell-Borlaug International Scholars Program for financial support. We also thank Stelle Proux-Wéra (SLU) for bioinformatic support through the PlantLink Network (SLU). We thank Jorge Montoya (CIMMYT) for assistance in conducting the field trial.

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Table 1. Main QTL location, LOD scores, additive effects and heritability for *Rhopalosiphum padi* antibiosis and *Schizaphis graminum* aphids/tiller.

QTL	Trait	Flanking markers	LG [†]	QTL interval (cM)	LOD score	Effect	PVE [‡] (%) by QTL	Heritability
<i>Rp1</i>	<i>R. padi</i> -antibiosis	TP48882; TP31989	4BL	31.1-36.0	3.1	-5.2	10.2	15.7
<i>Gba</i>	<i>S. graminum</i> /tiller	TP81905; <i>Gba</i>	7DL	116.1-119.8	5.9	-0.1	16.6	57.0
<i>QGb8</i>	???	TP67214; TP84201	2DL	71.7-74.8	3.9	-0.1	10.2	

[†]LG = Linkage group; [‡]Proportion of the total phenotypic variance explained by the QTL

Table 2. Main QTL location, LOD scores, additive effects, interaction effect and heritability for *Rhopalosiphum padi* tolerance.

QTL	Flanking markers	LG [†]	Interval (cM)	LOD	Effect	PVE [‡] (%) by QTL	Heritability
<i>Rp2</i>	TP3728; TP38148	5AL	106.5-107.0	3.7	-2.4	14.5	36.51
<i>Rp3</i>	TP3351; TP17691	5BL	143.2-148.9	3.8	-2.7	5.7	
<i>EnRp2</i>	TP62232; TP59798	3AL	99.7-100.2	0.35	0.6	6.7	
<i>Rp2*En Rp2</i>	TP3728*TP59798			3.1	1.6	5.0	

[†]LG = Linkage group; [‡]Proportion of the total phenotypic variance explained by the QTL

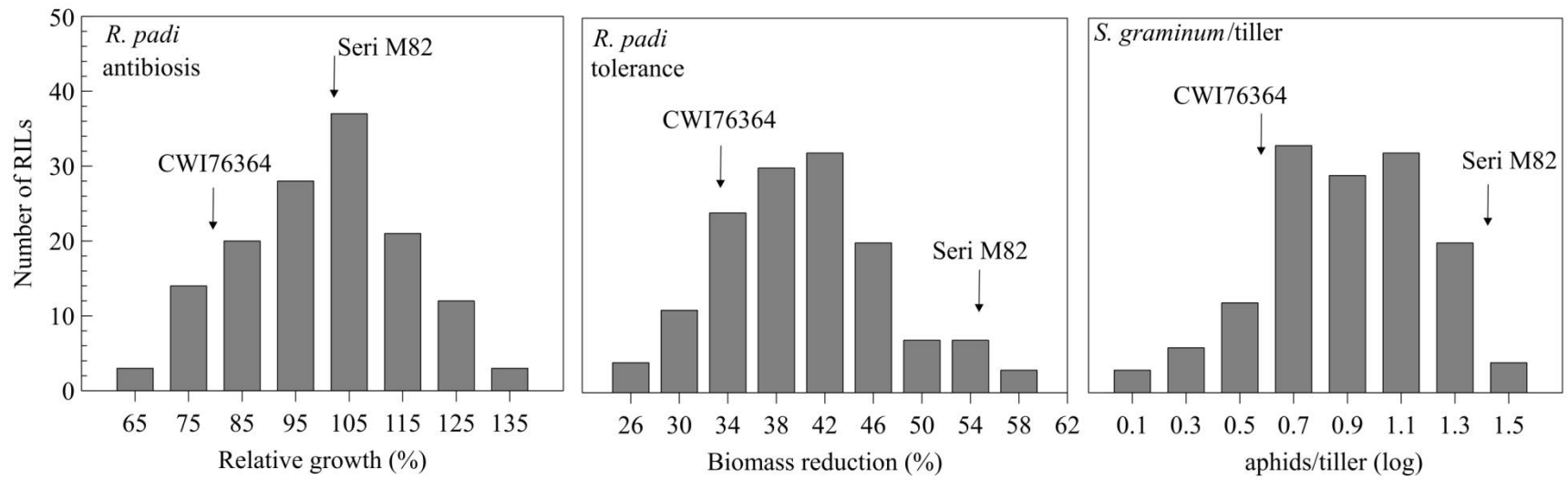


Figure 1. Histograms of the phenotypic response of the RILs to *R. padi* antibiosis, *R. padi* tolerance and *S. graminum*/tiller. The arrows indicate the phenotypic value of the two parents of the population.

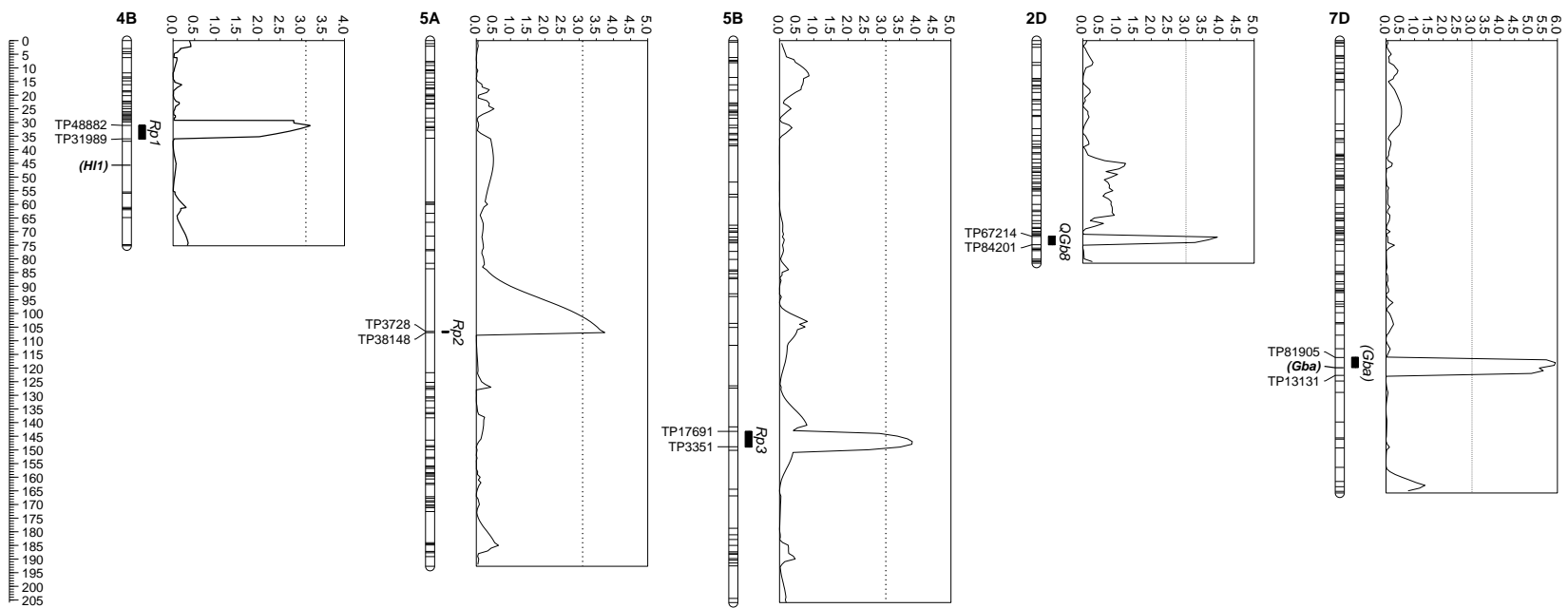


Figure 2. Linkage maps and LOD profiles of genomic regions associated to *R. padi* and *S. graminum* resistance in chromosomes 4B, 5A, 5B, 2D and 7D. The loci inside the parentheses indicate the putative genes.

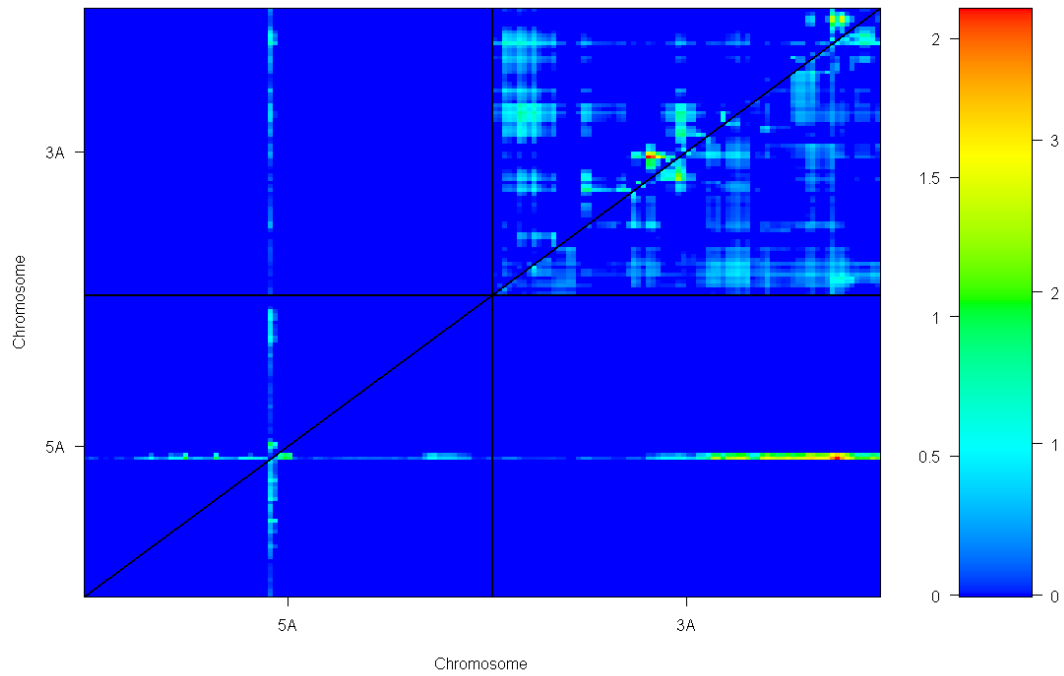


Figure 3. Heat plot of the 2-dimensional 2-qtl scan of chromosomes 5A and 3A. The triangle above the diagonal displays the LOD score of the model assuming only additive effects. The triangle beneath the diagonal displays the LOD scores of the model assuming that there is epistatic interaction between loci.

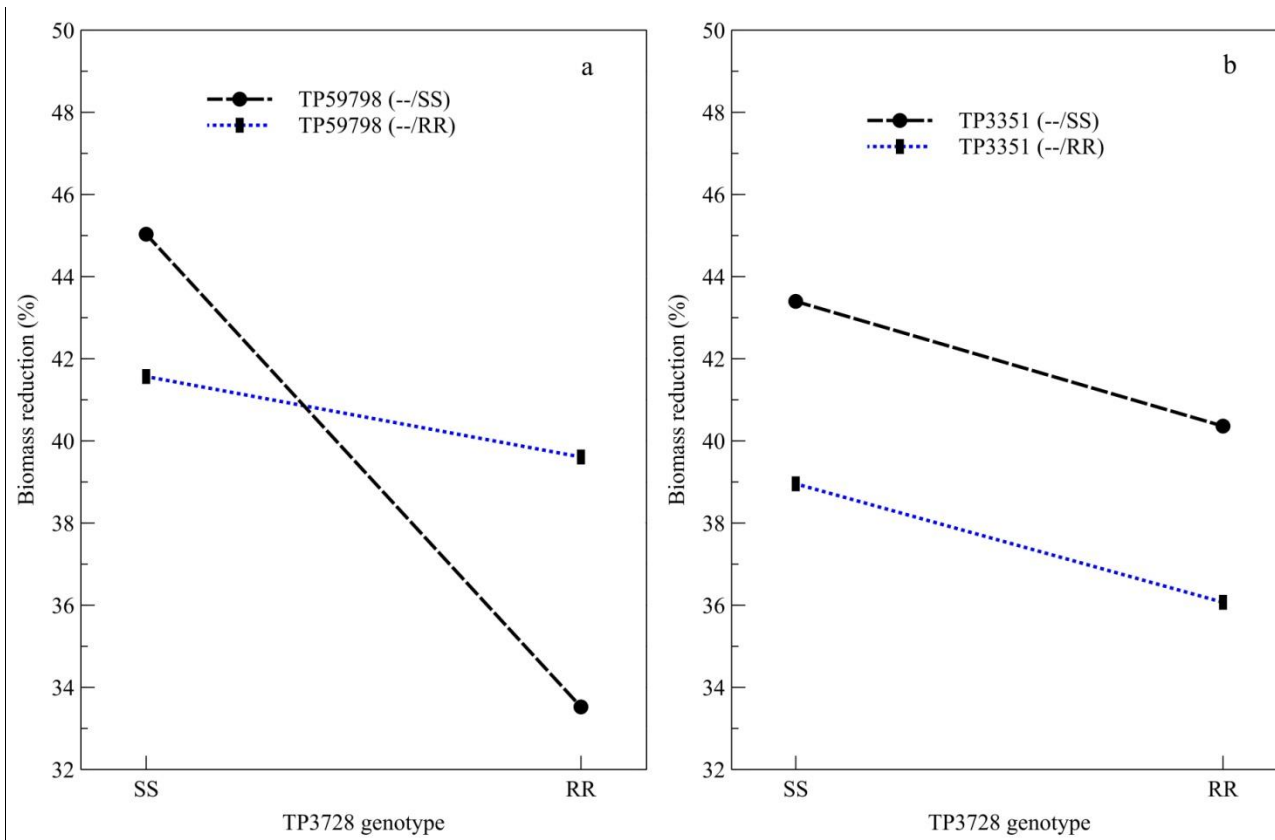


Figure 4. Interaction plots between, a) marker TP3728 linked to a main QTL in chromosome 5A and the epistatic locus linked to marker TP5978 in chromosome 3A, and b) Markers linked to main QTL effect displaying additive effect. Marker codes indicate whether alleles originate from the susceptible (SS) or resistant (RR) parent.

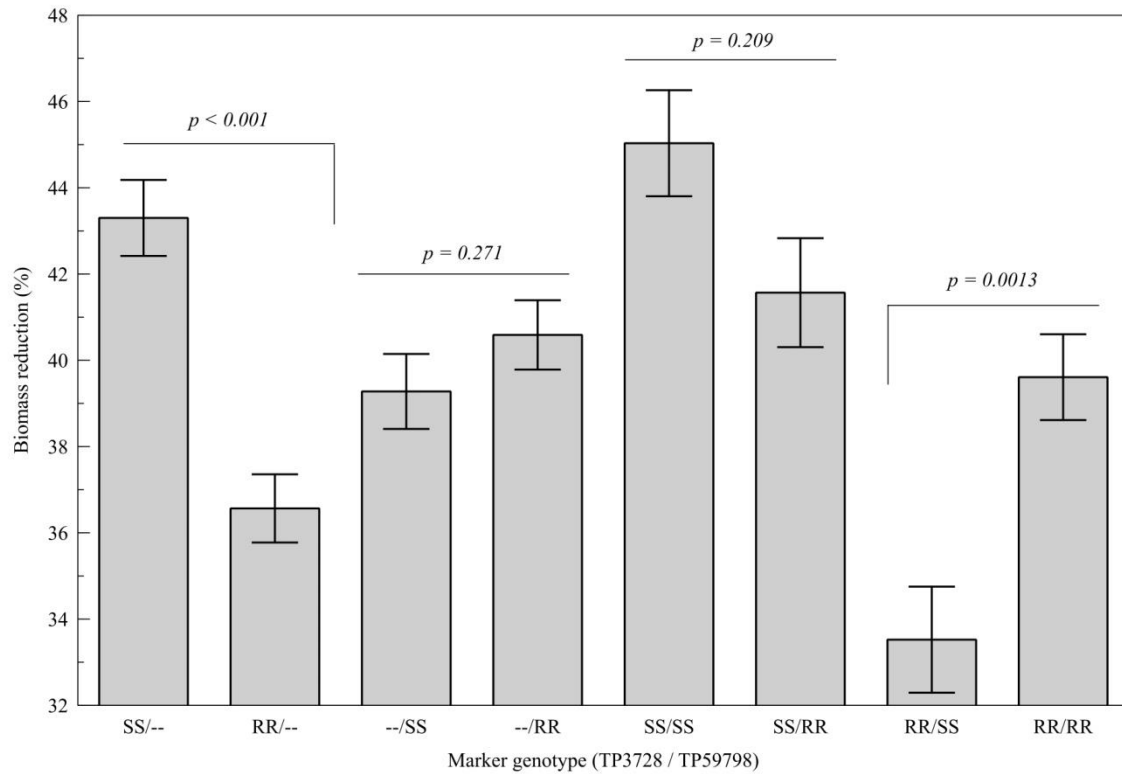


Figure 5. Phenotypic means and standard errors of markers linked to interacting loci associated the biomass reduction due to *R. padi* feeding. Marker codes indicate whether alleles originate from the susceptible (SS) or resistant (RR) parent. The *p-values* above the lines indicate the significance level in Tukey's tests for the pairs of means beneath the lines.