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ORIGINAL PAPER

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

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Abstract
Key message **Identification of novel resistance QTL against wheat aphids. First QTL-resistance report for *R. padi* in wheat and chromosome 2DL for *S. graminum*. These sources have potential use in wheat breeding.**
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 Seri M82 wheat (susceptible) with the synthetic hexaploid wheat CWI76364 (resistant). 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 the number of *S. graminum*/tiller in the field. RILs were also scored for pubescence. Using a sequence-based genotyping method, we located genomic regions associated with these resistance traits. A quantitative trait locus (QTL) for *R. padi* antibiosis (*QRp.slu.4BL*) that explained 10.2 % of phenotypic variation was found in chromosome 4BL and located 14.6 cM apart from the pubescence locus. We found no association between plant pubescence and the resistance traits. We found two QTLs for *R. padi* tolerance (*QRp.slu.5AL* and *QRp.slu.5BL*) in chromosomes 5AL and 5BL, with an epistatic interaction between a locus in chromosome 3AL (*EnQRp.slu.5AL*) and *QRp.slu.5AL*. These genomic regions explained about 35 % of the phenotypic variation. We re-mapped a previously reported gene for *S. graminum* resistance (putatively *Gba*) in 7DL and found a novel QTL associated with the number of aphids/tiller (*QGb.slu-2DL*) in chromosome 2DL. This is the first report on the genetic mapping of *R. padi* resistance in wheat and the first report where chromosome 2DL is shown to be associated with *S. graminum* resistance.

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Introduction

The aphids *Rhopalosiphum padi* L. and *Schizaphis graminum* (Rondani) are two of the most serious pests of wheat (*Triticum aestivum* L.), and can 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 the wheat-growing regions (Blackman and Eastop 2007). However, *S. graminum* is not a wheat pest in northern Europe. One effective strategy to reduce the damage caused by these pests is by breeding resistant varieties.

Selecting for resistance to these aphids in conventional wheat breeding programs is difficult to apply without sacrificing other breeding goals. Phenotypic selection requires considerable logistic efforts to maintain large segregating populations in various generations under homogenous aphid pressures across time and space. However, availability of molecular markers associated with aphid resistance genes can greatly facilitate the inclusion 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 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, one component often predominates over others to make a plant genotype more antibiotic, antixenotic or tolerant. Some complications of the categorization of insect resistance are: (a) antixenosis expressed as lower feeding rate may reduce insect performance and then be interpreted as antibiosis and (b) tolerance measured as reduced insect-specific plant symptoms may be due to reduced insect feeding and being unrelated 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 novel alleles that may confer resistance to various biotic stresses. One way to incorporate such variation in bread wheat is done with synthetic hexaploid wheats (SHW) that are derived 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 have been made since the 1950s (Porter et al. 1997; Berzonsky et al. 2003) and this has led to the identification of several resistance genes. Currently, 14 major genes have been identified for resistance to *S. graminum* in wheat (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 nine 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 (Burd and Porter 2006).

Despite the importance of *R. padi* as a pest, no *R. padi*-resistant wheat cultivars exist. Potential causes include the fact that *R. padi* foliar damage is less evident than that caused by other aphids, such as *S. graminum*, which causes measurable leaf chlorosis symptoms that enable selection of individual resistant plants. Additionally, the polyphagy and wide host adaptation of *R. padi* can make it difficult to find sources of resistance with adequate protection levels. These factors may also explain why no studies have been published on the genetics of *R. padi* resistance in wheat, and why no *R. padi* biotypes have been reported. Cheung et al. (2010) mapped *R. padi* resistance in barley, and reported a quantitative trait locus (QTL) in chromosome 3H that explains 9 % of the variation in aphid growth.

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. In a recently developed genotyping-by-sequencing (GBS) approach, the genome complexity is reduced by the use of restriction enzymes, followed by multiplex sequencing; it is thus possible to efficiently genotype a large number of lines (Elshire et al. 2011; Poland et al. 2012). This is particularly useful for species with complex genomes such as wheat, where sequencing needs to target non-duplicated regions of the genome to produce high-quality maps. GBS methods can be efficiently applied in bi-parental populations to map the genomic regions associated with various agronomic traits of interest (Saintenac et al. 2013).

In this study, we identified genomic regions associated with various traits related to resistance against *R. padi* and *S. graminum* in a SHW using a sequence-based genotyping approach (Saintenac et al. 2013). This is the first report on mapping antibiosis and tolerance to *R. padi* in wheat and the first report of *S. graminum* resistance in chromosome 2DL.

Materials and methods

Plant material

We developed a mapping population of 140 F_6 recombinant inbred lines (RILs) with the single-head descent method at

the International Maize and Wheat Improvement Center (CIMMYT) in Mexico. The parental lines 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* WX1027 (Lage et al. 2003). The CIMMYT´s *A. tauschii* accession WX1027 was also a progenitor of the population containing the *Gba* resistance gene 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).

R. 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 16 h of light, supplemented when needed with 400 W 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 three days and thereafter for two 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 16 h light at the intensity of 250 µmol photons m^{-2} s^{-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. Four seedlings of each RIL were randomly placed within each incomplete block.

When plants reached 2–3 leaf stage, they were individually exposed to five first instar nymphs that were born within a period of 24 h. These new-born nymphs were obtained from alate individuals previously caged on oat plants for 24 h. The 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 by the susceptible parent.

Tolerance of each RIL was measured as biomass reduction in an augmented split plot design experiment with infested and non-infested plant treatments. Each parent of the population was replicated 15 times. Biomass reduction was calculated with the formula: $1 - (I/\mathrm{NI})$, 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 × 61 × 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. Plants of the infested treatment were infested every second day with aphids at an approximate density of 45 aphids/plant during 15 days. Infestations started when seedlings were at the 2–3 leaf stage. Non-infested plants were treated with the systemic insecticide Confidor WG® (Bayer CropScience) at a dose of 3.5 % of active ingredient by pouring the solution into the soil (Dunn et al. 2007). One day after the last infestation, all plants were cut at the soil level and dried for 72 h 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.

S. 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 by courtesy of Dr. Gary Puterka, USDA-ARS Stillwater, OK USA and maintained on Jagger wheat. Biotype E is common and virulent to wheat carrying resistance genes *gb1* and *Gb2* (Burd and Porter 2006) but avirulent 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 randomized complete block design with four replicates. Plants were infested with an approximate density of 5 aphids/hill plot at the 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. A field test was conducted in this region at CIMMYT's Ciudad Obregon experimental station (27°37′N, 109°93′W) during the 2012–2013 winter season. The experiment was laid out in a rectangular row–column augmented design (6 × 30), with the population's parents replicated 12 times. The 140 RILs were sown on 20 December 2012, with 8–10 seeds of each RIL planted in a 10 cm hill plot in a bed-planting system with 0.8 m of distance between beds and 1 m 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 obtained 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 reached the 2–3 leaf stage.

Genotyping

Seedlings of the 140 RILs and the parents were grown in a greenhouse in the KSU Department of Plant Pathology. Plant tissue was collected when plants reached the 2–4 leaf stage. DNA extraction was performed using a QIAGEN DNeasy 96 Plant Kit®, according to the manufacturer instructions. DNA was quantified using the Quanti-iT™ PicoGreen® and concentrations were then normalized to 20 ng/µl.

GBS libraries were constructed following the protocol reported by Saintenac et al. (2013). The combination of PstI (barcoded adapter) and MseI (Y-adapter) restriction enzymes was used. We used a set of 96 barcodes (see supplementary file) with sticky ends complementary to the 3′ overhang of PstI and MseI-Y-adapters (Saintenac et al. 2013). Prior to the use of the adapters, the common primer (5′-CTCGGCATTCCTGCTGAACCGCTCTTCCGATCT-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 100 mM Tris–HCl and 500 mM NaCl buffer. Temperature was reduced from 95 to 30 °C in 65 cycles at a rate of 1 °C/min. Barcode adapters were then adjusted to a concentration of 0.2 µM.

DNA samples were separated 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; Saintenac et al. 2013). Restriction of DNA was made with a restriction mix that consisted of 20 µl of each DNA sample, 3.0 µl of 10× NEB Buffer 3, 0.5 µl of PstI (10 units), 1 µl of MseI (10 units) and 5.2 µl of H_2O, and 0.3 µl of 100× BSA. Digests were run for 3 h at 37 °C and then heated to 80 °C for 20 min. Ligation was performed by adding to the restriction digest (30 µl) of each sample, 3 µl of the unique barcode adapter, 8 µl of the Y-adapter and 9 µl of ligation Master Mix (2 µl of T4 DNA ligase buffer, 0.5 µl T4 DNA ligase (M0202L) and 6.5 µl water). Samples were then incubated at 22 °C for two hours and maintained at 65 °C for 20 min to inactivate the T4 DNA ligase. Before the amplification step, the samples were purified with a QIAGEN QIAquick PCR Purification Kit®. Then, 5 µl of each sample was pooled in one tube for each 96-well plate and eluted in 50 µl volume. Samples were amplified with 10 µM of PCR primers 5′-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT-3′ (forward) and 5′-CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAA-3′ (reverse) using a Taq 2X Master Mix from NEB (M0270L). There were 18 PCR cycles of 95 °C (30 s), 62 °C (10 s), 68 °C (20 s) that were terminated with a final extension at 72 °C for 5 min. The size and distribution of the DNA fragments in the genomic libraries were analyzed with the Bioanalyzer 7500 Agilent DNA Chip. Each DNA pool was sequenced in a single lane of 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, and identical reads were then grouped into tags. These tags were pairwise aligned to identify single base

pair mismatches, which represented candidate SNPs. Each pairwise alignment represented a node in a network. The complex networks and those without a single reciprocal mismatch were discarded with a network filter at an error rate tolerance of 0.03 to provide the reciprocal tag pairs used for SNP calling. After processing the Illumina Fastq files with the UNEAK pipeline, a total of 1,313 GBS markers were used for the QTL analysis.

Linkage groups (LG) were constructed using 1,313 GBS markers, the categorical responses of each RIL to *S. graminum* leaf damage, and plant pubescence scores. The IciMapping software (Li et al. 2007) was used to group the markers with a LOD score of nine. Markers were ordered with the Traveling Salesman algorithm, using a 5 cM window size for rippling LG. Linkage groups with less than three markers or markers with no linkage were discarded from the analysis (altogether 3 out of 25). To assign 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 EnsamblPlants (http://plants.ensembl.org/Triticum_aestivum/Info/Index). Sequences of the GBS markers in each linkage group were entered into the search engine of EnsamblPlants in FASTA format and the chromosomes were assigned given the scaffold where the sequences were aligned.

The IciMapping software 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 1,000 permutations and a 0.001 probability value for markers to enter the QTL model (Da Costa E Silva et al. 2012a, b). Linkage groups and QTLs were plotted with MapChart software (Voorrips 2002). Parental sequences of GBS markers linked to QTLs are given in supplementary Table 2.

To determine if QTLs associated with *R. padi* tolerance were related to plant growth per se, IciMapping was also used to conduct a multi-environmental QTL analysis. For this analysis, the data from the infested and non-infested treatments were considered as different environments to locate QTL associated with biomass production per se across both treatments.

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 and Sen 2009; 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 1,000 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 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 phenotypic values given by the marker genotypes.

Results

There were 22 LG used for the analysis, spanning a total length of 2252.7 cM, based on 1,309 GBS markers. Thus, there was an average of one marker for 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 chromosome arm 4DL. Out of the 1,309 GBS markers, 36.0, 29.3 and 34.6 % were mapped in the A, B and D genomes, respectively.

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 ($k_3 = -0.1$) (Fig. 1). The heritability estimate was 0.157 (Table 1). A genomic region significantly associated with *R. padi* antibiosis was identified in chromosome 4BL, and was flanked by markers TP48882 and TP31989 within an interval of 4.9 cM (Fig. 2). This region explained 10.2 % of the total phenotypic variation (Table 1). Hereafter, this locus will be referred to as *QRp.slu-4BL*. The TP48882 marker was 0.1 cM from *QRp.slu-4BL*.

The genomic region associated with 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 ($df = 1$; $\chi^2 = 3.55$; $p = 0.06$), 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. No epistatic interactions were found in the two-dimensional scan.

Tolerance to *R. padi*

The phenotypic data for *R. padi* tolerance did not deviate from a normal distribution according to the

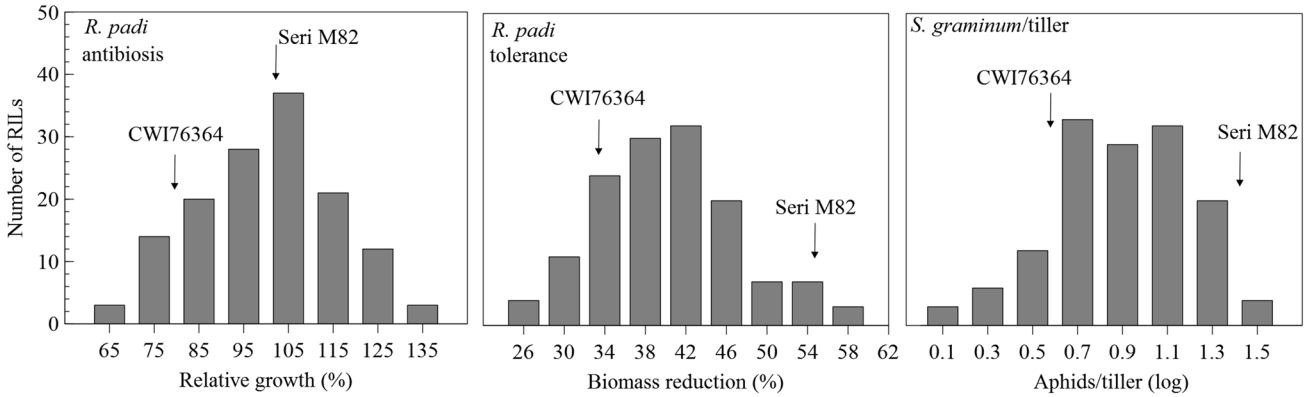

Fig. 1 Histograms of the phenotypic response of the RILs to *R. padi* antibiosis, *R. padi* tolerance and *S. graminum*/tiller. The *arrows* indicate the phenotypic value of the two parents of the population

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

QTL	Trait	Flanking markers	LG[a]	Interval (cM)	LOD score	Effect	PVE[b] (%) by QTL
QRp.slu-4BL	*R. padi*-antibiosis	TP48882-TP31989	4BL	31.1–36.0	3.1	−5.2	10.2
Gba	*S. graminum*/tiller	TP81905-*Gba*	7DL	116.1–119.8	5.9	−0.1	16.6
QGb.slu-2DL	*S. graminum*/tiller	TP67214-TP84201	2DL	71.7–74.8	3.9	−0.1	10.2

[a] *LG* Linkage group, [b] Proportion of the total phenotypic variance explained by the QTL

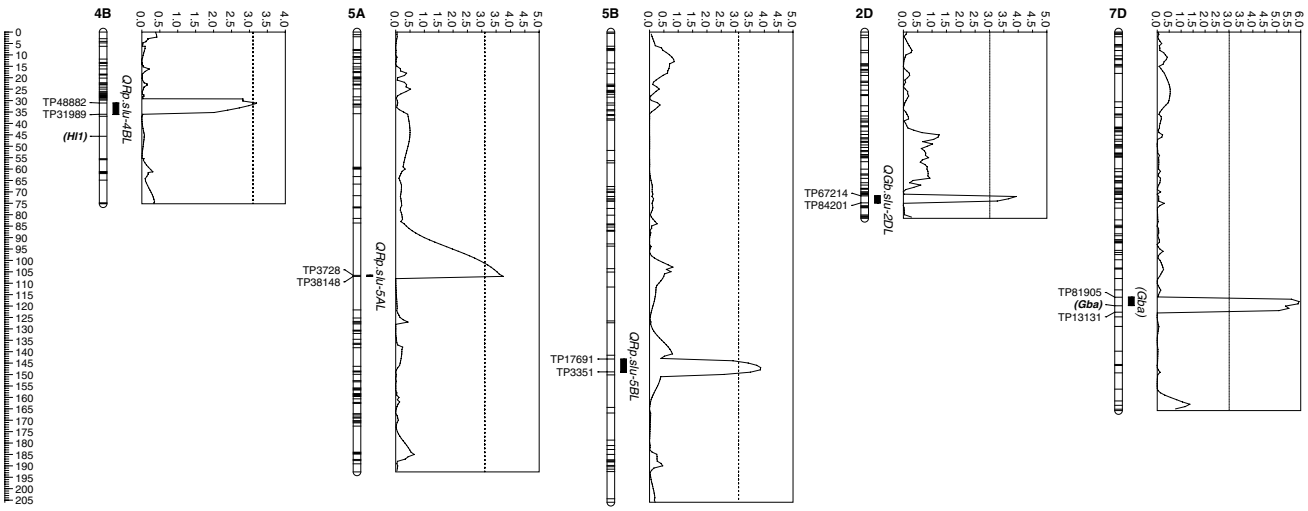

Fig. 2 Linkage maps and LOD profiles of genomic regions associated with *R. padi* and *S. graminum* resistance in chromosomes 4B, 5A, 5B, 2D and 7D. The loci within *parentheses* indicate the putative genes

Anderson-Darling test ($A^2 = 0.45$; $p > 0.25$) but appeared to be right-tailed ($k_3 = 0.33$) (Fig. 1). The estimated heritability for this trait was 0.365. Two significant QTLs associated with *R. padi* tolerance that were found in chromosomes 5AL and 5BL explained 14.5 and 5.7 % of the phenotypic variation, respectively (Table 2). The closest markers to these genomic regions are TP3728 and TP3351, respectively (Fig. 2). Hereafter, these loci will be referred to as *QRp.slu-5AL* and *QRp.slu-5BL*, respectively.

The two-dimensional scan revealed one epistatic locus present in chromosome 3AL interacting with the marker TP3728 associated with *QRp.slu-5AL* (Fig. 3). The epistatic locus is closest to the marker TP59798, and herein referred to as *EnQRp.slu-5AL*. The interaction accounted

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

QTL	Flanking markers	LG[a]	Interval (cM)	LOD	Effect	PVE[b] (%) by QTL
QRp.slu-5AL	TP3728-TP38148	5AL	106.5–107.0	3.7	−2.4	14.5
QRp.slu-5BL	TP3351-TP17691	5BL	143.2–148.9	3.8	−2.7	5.7
EnQRp.slu-5AL	TP62232-TP59798	3AL	99.7–100.2	0.35	0.6	6.7
*QRp.slu-5AL*EnQRp.slu-5AL*	TP3728*TP59798			3.1	1.6	5.0

[a] *LG* Linkage group, [b] Proportion of the total phenotypic variance explained by the QTL

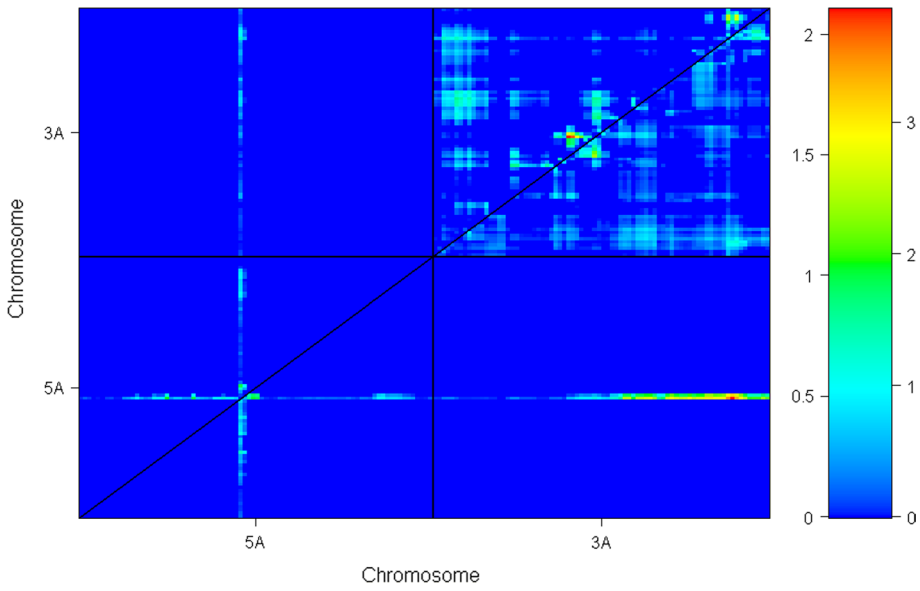

Fig. 3 Heat plot of the 2-dimensional, 2-qtl scan of chromosomes 5A and 3A. The *triangle* above the diagonal displays the LOD score of the model assuming only additive effects. The *triangle* beneath the diagonal displays the LOD scores of the model assuming that there is epistatic interaction between loci. Left and right sides of the *scale bar* correspond to LOD scores of the *triangle* above and beneath the diagonal, respectively

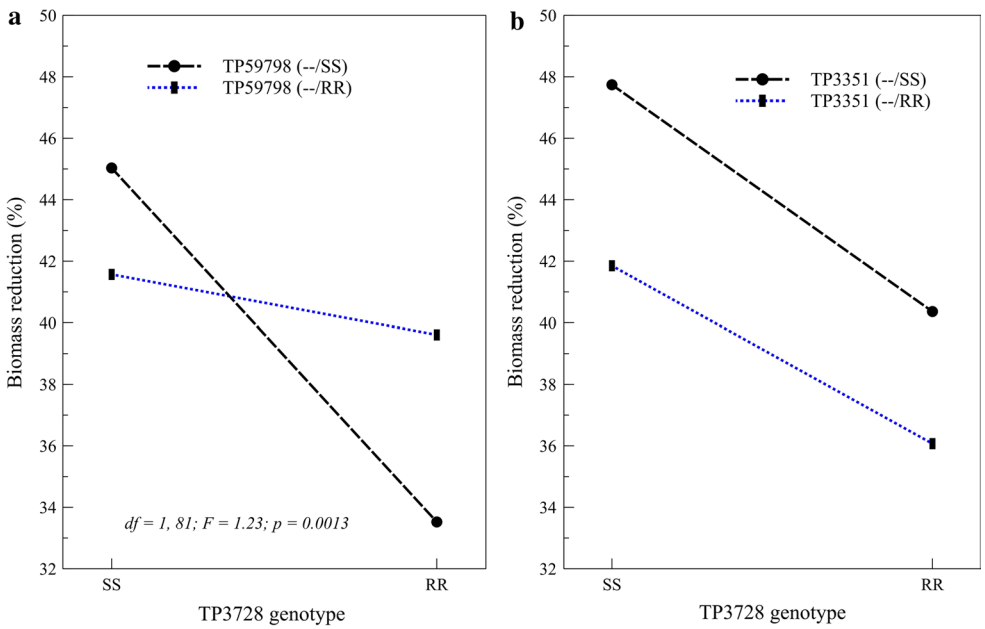

Fig. 4 Interaction plots between, **a** marker TP3728 linked to a main QTL in chromosome 5A and the epistatic locus linked to marker TP59798 in chromosome 3A, and **b** markers linked to main QTL effect displaying additive effect. *Marker codes* indicate whether alleles originate from the susceptible (SS) or resistant (RR) parent

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 also showed that the interaction TP3728 * TP59798 was significant ($df = 1, 81$; $F = 16.26$; $p = 0.0001$; Fig. 4a),

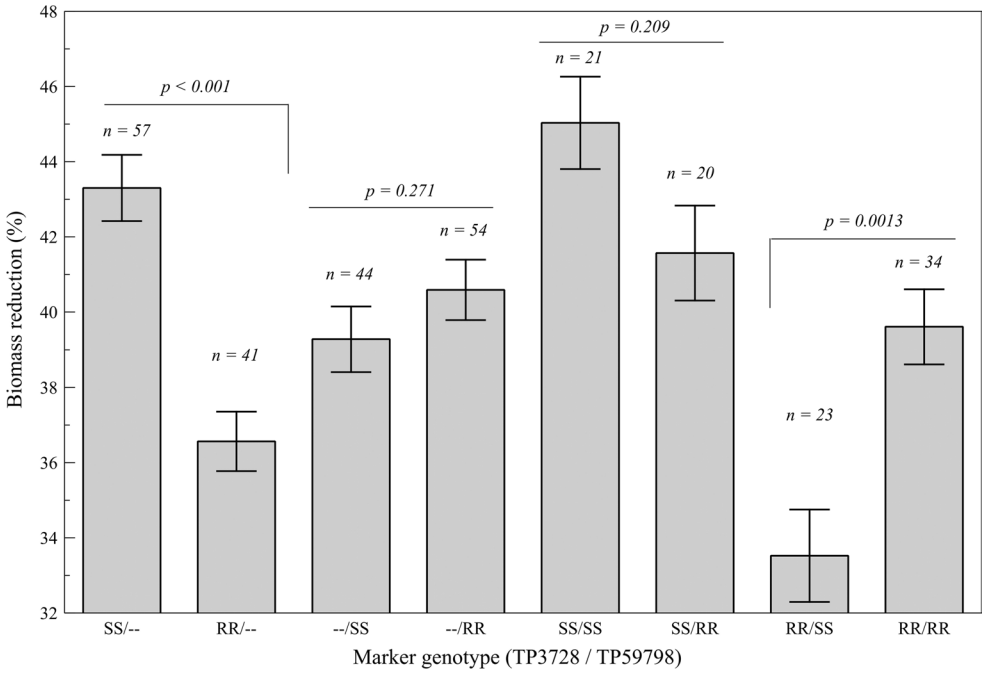

Fig. 5 Phenotypic means of RILs grouped according to the genotype of markers linked to interacting loci associated with the wheat biomass reduction due to *R. padi* feeding. *Marker codes* indicate whether alleles originate from the susceptible (*SS*) or resistant (*RR*) parent. The *p values* above the lines indicate the significance level in Tukey's tests for the pairs of means beneath the lines. n = RILs

while there was no effect of the TP59798 marker alone ($df = 1, 81$; $F = 1.23$; $p = 0.2721$). Figure 4b shows the additive effect of *QRp.slu-5AL* and *QRp.slu-5BL*. The comparison of means for all combinations of these marker genotypes showed that the level of biomass reduction was lowest when *QRp.slu-5AL* (TP3728) from the resistant parent combined with the allele from the susceptible parent of marker TP59798 (Fig. 5).

The multi-environmental QTL analysis across infested and non-infested treatments showed no common QTL between plant growth per se and *R. padi* tolerance; therefore, these results are 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 appeared to be left-tailed ($k_3 = -0.3$) (Fig. 1). The estimated heritability was 0.57. Greenhouse phenotyping for *S. graminum*-induced chlorosis showed that 55.7 % of the RILs were resistant and 44.3 % were susceptible. These values did not deviate from the expected segregation ratio of 1:1 ($df = 1$; $\chi^2 = 1.82$; $p = 0.17$) for a single locus, most likely the gene *Gba* previously mapped by Zhu et al. (2005) in chromosome 7DL. Our linkage analysis also placed this locus in chromosome 7DL, between markers TP81905 and TP13131 (Fig. 2), and was also significantly associated with the number of *S. graminum*/tiller in the QTL analysis, explaining 16.6 % of the phenotypic variation (Table 1).

A genomic region in chromosome 2DL was also associated with the number of *S. graminum*/tiller, explaining 10.2 % of the phenotypic variation (Table 1; Fig. 2). The QTL model for aphids/tiller explained 29.1 % of the total phenotypic variation. The two-dimensional scan showed no 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 with 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). Additionally, in our study, GBS markers appeared to be distributed almost equally among the three wheat genomes, which indicate a homogenous coverage. Here, we applied GBS for fast mapping of known and novel genomic regions associated with resistance to two aphid species that are important pests of wheat.

Despite presenting some degree of skewness, all phenotypic responses fit a normal distribution, 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, which had a low heritability estimate. However, low heritability is a common feature of insect life history traits, since they are highly influenced by the environment (Price and Schluter 1991).

Antibiosis to *R. padi*

We found one genomic region associated with *R. padi* antibiosis (*QRp.slu-4BL*) and a plant pubescence locus in the same chromosome. However, they were located 14.6 cM apart from each other, 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 leaf trichomes. In our study, neither *R. padi* nor *S. graminum* resistance was related to such a plant trait, indicating that *R. padi* antibiosis and numbers of *S. graminum*/tiller are caused by mechanisms different from pubescence in the resistant genotype CWI76364. No other published reports have investigated genetic associations between aphid resistance and plant pubescence in wheat. It is probable that the pubescence locus we found is allelic to, or the same as the hairy leaf gene *Hl1* previously mapped 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.

Furthermore, chromosome 4BL also harbors genes (*Tbx*) involved in the synthesis of hydroxamic acids (hx) (Nomura et al. 2002; Nomura et al. 2003). Hx play an important role in the defense of gramineous plants against herbivores, causing antibiotic effects on insects including various aphid species (Frey et al. 1997; Niemeyer 2009). The hx DIBOA and DIMBOA are present in hexaploid wheat; however, only the latter is present in high concentrations (Niemeyer et al. 1992). Niemeyer et al. (1992) found that *Aegilops speltoides* Tausch, the most likely donor of the B genome (Dvorak and Zhang 1990), possesses higher concentrations of DIMBOA when compared with the other wheat genome donors. This suggests that the B genome is responsible for the synthesis of high concentrations of hx in hexaploid wheat. It would be of great interest to determine whether *QRp.slu-4BL* is associated with the *Tbx* genes located in the proximal section of 4BL by analyzing the DIMBOA concentrations in our population.

Tolerance to *R. padi*

Two genomic regions associated with wheat seedling tolerance to *R. padi* were found in chromosomes 5AL and 5BL (*QRp.slu-5AL* and *QRp.slu-5BL*, respectively). These genomic regions are related to biomass reduction under *R. padi* stress and not to biomass production per se, suggesting that expression of *QRp.slu-5AL* and *QRp.slu-5BL* may be induced by aphid feeding.

In addition, part of the genetic effect of *R. padi* tolerance was explained by epistasis. The implementation of the two-dimensional scan displayed a significant epistatic interaction for *R. padi* tolerance that changed the magnitude of *QRp.slu-5AL*. This interaction is given by a genomic region (*EnQRp.slu-5AL*) located in chromosome 3AL, linked to the marker TP59798. Tolerance to *R. padi* appears to be enhanced when the marker allele of TP59798 from the susceptible parent Seri M82 is present along with *QRp.slu-5AL*. The epistatic locus *EnQRp.slu-5AL* had in itself no significant effect on biomass reduction due to *R. padi* feeding. The conditions for identification of epistatic interaction are optimal in bi-parental mapping populations because allele frequencies tend to be equal to 0.5 and thus the epistatic variance is maximized. Additionally, 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, phenotyping entails stringent conditions that guarantee that plants of each genotype have approximately the same starting size in both treatments. Additionally, to rule out antibiosis and/or antixenosis effects, plants must be supplied with repeated aphid inoculations. However, once proven to be of significant importance for biomass and eventually seed production, molecular markers such as those provided by our study will 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.

Tolerance is an attractive component of insect resistance, as it poses no selection pressure on the insect pest, minimizing the risk of virulence development compared with a scenario where insects are under constant selection due to antixenosis or antibiosis. Tolerance may facilitate other aphid control methods, since insecticide treatments in commercial farms often occur after aphid populations have exceeded the economic threshold on a susceptible cultivar. A tolerant cultivar may endure high aphid populations for a long enough period to localize the infestation and if necessary apply a control measure.

S. graminum resistance

Phenotyping of *S. graminum* resistance is often based on the levels of leaf chlorosis due to aphid feeding, as reduced chlorosis might be interpreted as plant tolerance. Results from a microarray study comparing plants with and without *S. graminum* support this (Reddy et al. 2013). Aphid-infested plants carrying *Gb3* prevent cell wall modification and consequently cell death, and also down-regulate genes

for the synthesis of secondary metabolites, presumably saving plant resources (Reddy et al. 2013). The tolerance component of *Gba* plants is likely explained by similar molecular mechanisms. However, resistance by *Gb3* has also been characterized as antixenosis and antibiosis effects on *S. graminum* biotype E (Weng et al. 2004). This may explain the lower number of aphids/tiller that we found in the field, in our case putatively caused by *Gba.*

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

Additionally, we found a novel genomic region in chromosome 2DL to be associated with the frequency of *S. graminum*/tiller in the field. This QTL, here referred to as *QGb.slu-2DL,* contributed to 10.2 % of the phenotypic variation. We hypothesize that this 2DL region was unreported in *A. tauschii* WX1027 because it was associated with aphids/tiller in the field, whereas Zhu et al. (2005) evaluated symptoms based on chlorosis in a greenhouse test similar to ours, where we also found no association with 2DL. It is possible that *QGb.slu-2DL* has a predominantly antixenotic effect on *S. graminum* since Lage et al. (2003) showed that CWI76364 was the most antixenotic among 12 studied SHWs. However, reduced aphids/tiller could also be due to antibiosis since this SHW is more antibiotic than the susceptible cultivar Seri M82 (Lage et al. 2003). Another possible explanation for the new QTL for *S. graminum* resistance might be an uncharacterized biotype in our field experiment, since we did not determine the biotypes of *S. graminum*. However, Burd and Porter (2006) reported that biotype E and I are the most common in wheat in the USA. Further investigation is required to confirm and characterize *QGb.slu-2DL* and determine the type of resistance it confers against *S. graminum*.

Conclusions

We utilized sequence-based genotyping to determine the genetic bases of resistance to *R. padi* and *S. graminum* in the synthetic hexaploid wheat CWI76364. Results identified one locus for *R. padi* antibiosis (*QRp.slu-4BL*) in chromosome 4BL and two loci for *R. padi* tolerance (*QRp.slu-5AL* and *QRp.slu-5BL*) in chromosomes 5AL and 5BL, respectively. An allele originating from the susceptible parent (*EnQRp.slu-5AL*) was also identified to enhance the effect of *QRp.slu-5AL*. From these results, we conclude that resistance to *R. padi* originates from *T. dicoccum* in the CWI76364/Seri M82 population. This is the first report of genetic mapping of antibiosis, tolerance and epistatic effects against *R. padi* in hexaploid wheat. Using GBS, we re-mapped the putative *Gba S. graminum* resistance gene, and identified a genomic region (*QGb.slu-2DL*) in chromosome 2DL associated with numbers of *S. graminum*/tiller in the field. Plant pubescence was unrelated to any resistance traits measured for either aphid species. The identification of the GBS markers associated with aphid resistance loci will help fine map those genomic regions and develop new molecular markers that are easier to apply in wheat breeding. The loci we have discovered are promising sources to be deployed in elite wheat gemplasm, since the combination of several resistance mechanisms should make the resistance more durable. Nonetheless, more efforts to find and characterize additional sources of aphid resistance are needed to further enhance wheat 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 contribute to a more environment-friendly production of wheat.

Author contributions C-HLA: R. *padi* and S. *graminum* phenotyping, genotyping, SNP calling and data analysis; AE, JKW, GGL: genotyping, SNP calling; SCM: S. *graminum* phenotyping; SRP: S. *graminum* phenotyping, germplasm development; IÅ: R. *padi* phenotyping, data analysis. C-HLA wrote the manuscript. All authors read, contributed to manuscript preparation and approved the final version.

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

Ethical standards The experiments comply with the current laws of the countries in which they were performed.

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