Fine mapping and comparative genomics integration of two quantitative trait loci controlling resistance to powdery mildew in a Spanish barley landrace

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Abstract

The intervals containing two major quantitative trait loci (QTL) from a Spanish barley landrace conferring broad spectrum resistance to *Blumeria graminis*, were subjected to marker saturation. First, all the available information on recently developed marker resources for barley was exploited. Then, a comparative genomic analysis of the QTL regions to other sequenced grass model species was performed. As a result of the first step, thirty-two new markers were added to the previous map and new flanking markers closer to both QTL were identified. Next, syntenic integration revealed that the barley target regions showed homology with regions on chromosome 6 of rice (*Oryza sativa*), chromosome 10 of *Sorghum bicolor* and chromosome 1 of *Brachypodium distachyon*. A nested insertion of ancestral syntenic blocks on Brachypodium chromosome 1 was confirmed. Based on sequence information of the most likely candidate orthologous genes, twenty-three new barley unigene-derived markers were developed and mapped within the barley target regions. The assessment of colinearity revealed an inversion on chromosome 7HL of barley compared to the other three grass species and nearly perfect colinearity on chromosome 7HS. This two-step marker enrichment allowed for the refinement of the two QTL to much smaller intervals. Inspection of all predicted proteins for the barley unigenes identified within the QTL intervals did not reveal the presence of resistance gene candidates. This study demonstrates the usefulness of sequenced genomes for fine mapping and paves the way for the use of these two loci in barley breeding programs.

Key words: comparative genomics, barley powdery mildew resistance, marker saturation, candidate genes

Introduction

Albeit the numerous efforts in the past decades to identify and characterize new resistance genes, *Blumeria graminis* still ranks high among barley pathogens worldwide due to its adaptability and ability to cause severe yield losses (McDonald and Linde 2002). Therefore, barley breeders need new resistance genes or alleles effective against the virulence spectra arising in the existing pathogen populations. A strategy that has been proposed to increase the durability of resistance consists in pyramiding several resistance genes in a single cultivar (Friedt et al. 2003). This strategy specifically demands for the use of molecular markers. Indeed, any introgression scheme is made more effective by the use of marker technology (Varshney et al. 2006a). An efficient introgression of new resistance genes from un-adapted germplasm demands perfect markers within the target loci or, at least, so-called diagnostic markers, tightly linked to the trait of interest (Perovic et al. 2009). The development of efficient markers is facilitated by a rapidly growing array of genomic resources and, in many cases, even the full sequence of the target species. The advent of complete sequence information will reshape research approaches in plant genetics. Although, previoussequencing activities (e.g. Wicker et al. 2006; Mayer et al. 2009), have been hampered due to the large genome size and the presence of large fractions of repetitive DNA, the firts draft sequences of Morex and Betzes opened a new era to barley genetics (Wicker et al 2011) . However, searching for genes in barley will still rely on synteny with related species (Mayer et al. 2011). This study describes a practical approach to develop a high resolution map for two disease resistance loci using genomic resources developed for barley and other grasses.

In barley, a major focus has lately been put on the generation of expressed sequence tag-based (EST-based) molecular markers. More than 500,000 ESTs generated from different cDNA libraries are deposited at NCBI (http://www.ncbi.nlm.nih.gov/dbEST/), providing an excellent source for developing DNA markers (Rostoks et al. 2005; Varshney et al. 2006b; Marcel et al. 2007). The first comprehensive barley transcript map was developed by Stein et al. (2007), using more than 1,000

EST-derived markers. More recently, two high density transcript linkage maps of barley were reported by Sato et al. (2009) and Close et al. (2009), each one containing more than 2,500 loci. These maps offer great opportunities for increasing the marker density on promising chromosomal regions.

Several grasses with complete genome sequence, namely rice (*Oryza sativa*), sorghum (*Sorghum bicolor*) and *Brachypodium distachyon* (subsequently Brachypodium), have been proposed as models for molecular genomics of cereals with large genomes. The model based strategy is supported by the extensive conservation of gene content -synteny- among the Poaceae genomes (Bolot et al. 2009; Abrouk et al. 2011). Since the release of the rice genome (Goff et al. 2002; Yu et al. 2002), colinearity between rice and barley has been widely exploited to develop EST-based markers for saturation mapping of different loci (Perovic et al. 2004; Mammadov et al. 2005; Komatsuda et al. 2007; Chen et al. 2009; Ramsay et al. 2011; among others), to identify putative orthology/paralogy of multigene family members (Faure et al. 2007; Perovic et al., 2007) and to mine for candidate genes (Stein et al. 2005). Regarding marker saturation, a significant level of colinearity has been demonstrated for large regions of barley chromosome 7H and rice chromosome 6 (Thiel et al. 2009; Druka et al. 2011). Barley synteny studies involving sorghum and Brachypodium, which have been sequenced recently (Paterson et al. 2009; International Brachypodium Initiative 2010), are still rare, although the number is increasing. Among these recent reports, the wild grass Brachypodium has emerged as the most important model for wheat and barley (Bossolini et al. 2007; Faris et al. 2008; Chen et al. 2009; Drader and Kleinhofs 2010; Higgins et al. 2010). Synteny between species is not equally conserved across genomic regions. For instance, Turner et al. (2005) used synteny with Brachypodium to locate an orthologous gene in barley, because local synteny with rice in the region was poor. Therefore, any approach using synteny approaches to search for genes in barley (or any species lacking a reference sequence) may benefit from complementary information contributed by surveying several related species.

The loci targeted in this study derive from a largely untapped reservoir of genetic diversity, i.e. the Spanish Barley Core Collection (SBCC) (Igartua et al. 1998). It has been evaluated for a wide range of agronomic traits, including resistance to a variety of fungal and viral diseases by Silvar et al. (2010a). This study revealed promising levels of resistance to the fungal pathogen *Blumeria graminis*, the causal agent of powdery mildew. A more exhaustive analysis of powdery mildew resistance in Spanish barleys revealed that some landrace-derived inbred lines might possess novel genes or alleles that have not been identified in other barley resources (Silvar et al. 2011a). The resistances from the two most interesting lines were investigated further in mapping populations, resulting in the identification of different QTL (Silvar et al. 2010b, Silvar et al. submitted).

The novel resistance loci detected in Spanish landraces may be valuable for barley breeding programs, given their broad spectra of resistance. This will be especially true if they are pyramided with other genes or QTL against *B. graminis*. For this purpose, the knowledge of either the candidate gene or at least closely linked markers is needed. In previous work, two QTL for resistance to powdery mildew were identified in the Spanish barley landrace-derived line SBCC097 (Silvar et al. 2010b). The first locus was located on chromosome 7HS in a 15 cM interval. The second QTL mapped to 7HL, but was located outside the most distal telomeric marker of the linkage group (EBmac0755), which added uncertainty to its position. The marker density in the QTL intervals at that time did not allow for efficient marker assisted selection, less to say map-based cloning. The main goals of the present study were, therefore, (i) to employ EST-derived markers from previously published sources for marker enrichment of the QTL regions of chromosome 7H in the SBCC097×Plaisant population and (ii) to conduct genomic comparisons between barley, rice, sorghum and Brachypodium within this region to refine the position of the QTL and develop more tightly linked DNA markers for the resistance loci, paving the way for future map-based cloning of the genes involved.

Materials and Methods

Plant and pathogen materials

The SBCC097×Plaisant F_5 and F_6 RIL population (262 lines) was used to select 94 lines showing recombination between flanking markers GBM1126/GBM1060 (7HS QTL) and markers GBM1120/EBmac0755 (7HL QTL) (Silvar et al. 2010b, Fig. 1A). Out of these 94 lines, 46 were selected as the most informative ones, for their clear-cut phenotypic responses, and for the unequivocal presence of just one of the two QTL on 7H, based on marker information.

Three barley reference doubled haploid (DH) populations: Igri×Franka (I×F) (Graner et al. 1991), Steptoe×Morex (S×M) (Kleinhofs et al. 1993) and Oregon Wolfe Barley OWB-D×OWB-R (D×R) (Costa et al. 2001) were used for the selection of GBR, GBM and GBS markers at the target intervals. Phenotypic data on the disease scores of four *B. graminis* isolates (R79, R180, R126 and R178) after infection of the SBCC097×Plaisant RIL population are available from a previous work (Silvar et al. 2010b).

Marker development and genotyping

Previously published barley genetic maps of EST-based markers were surveyed to select potential markers at the target intervals on chromosome 7H (Stein et al. 2007; Marcel et al. 2007; Sato et al. 2009).

The three barley reference populations described above were screened with both sets of QTL flanking markers; GBM1126/GBM1060 and GBM1102/EBmac0755. Then, GBR (RFLP), GBM (microsatellite) and GBS (SNP) markers from Stein et al. (2007) were selected within the QTL intervals based on single recombination data. The sequences of the primer pairs and amplification protocols for microsatellite markers (GBM) and SNP based markers (GBS) were obtained from Stein et al. (2007). GBR markers were sequenced and converted into PCR based markers. For this purpose, publicly available genomic sequence information in GrainGenes

(http://wheat.pw.usda.gov/) and GenBank (http://www.ncbi.nlm.nih.gov/) was employed to design primers for amplification of about 400 bp fragments from genomic DNA of 'SBCC097' and 'Plaisant'. Primers were designed using Primer 3 software (Rozen and Skaletsky 2000). Newly designed PCR-markers derived from RFLP-GBR markers were named as QBS, standing for Quedlinburg Barley SNP.

Selected markers from Sato et al. (2009) were directly amplified with primers designed by those authors, and the PCR fragment of both parents was sequenced. Therefore, markers from Sato et al. (2009) were not renamed, although the SNPs found in the present work were different from those reported by Sato et al. (2009).

Simultaneously, 46 out 94 recombinant lines were genotyped with the 1,536-SNP Illumina GoldenGate Oligonucleotide Pool Assay (BOPA1) (Close et al. 2009) at the Southern California Genotyping Consortium at UCLA.

Routine PCR was done in 10 µl reaction volume including 25–50 ng genomic DNA, 0.5 U of Taq DNA Polymerase (Solis Biodyne, Tartu, Estonia) 1×PCR reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs and 0.2 µM of each primer. All fragments were amplified using the following touch-down PCR profile: an initial denaturing step of 5 min at 94°C was followed by 35 cycles with denaturation at 94°C for 30s and extension at 72°C for 30s, respectively. The annealing temperature was decreased in 0.5 °C increments from 62ºC in the first cycle to 56 °C and was then kept constant for the remaining 35 cycles (always 30-50s). A final extension step was performed at 72 °C for 10 min.

Purified amplicons were subjected to cycle-sequencing from both ends on the ABI377XL sequencers using BigDye v3.1 terminator sequencing chemistry (ABI Perkin Elmer, Weiterstadt, Germany). Sequence analysis and identification of polymorphisms were conducted using the free-trial version of the software package Geneious Pro (Drummond et al. 2007; available at www.geneious.com). The presence of restriction sites at SNP positions was analysed with the

NEBcutter v.2.0 tool (http://tools.neb.com/NEBcutter2). Sequence polymorphisms between the parents were transformed to either CAPS (Cleaved Amplified Polymorphism) markers or pyrosequencing markers. Restriction digestion of PCR products were carried out in a 15 µl volume using 1U of the respective restriction endonuclease (NEB, Fermentas) and an incubation time of 3 hours. The restricted fragments were separated in 1.8 % agarose gel. When there was no restriction enzyme available for the SNP, a pyrosequencing assay was developed on a PyroMark ID system (Biotage). The experimental procedure for the pyrosequencing assays, including PCR optimization, was done by using an M13 universal primer labelled with biotin (Silvar et al. 2011b). Genotyping of SNP markers from BOPA1 (Close et al. 2009) on additional 48 RILs was performed by using the same pyrosequencing approach.

Comparative genomic analysis

In order to define more accurately the regions including the resistance loci, we developed new markers based on the synteny of the barley genome with rice, sorghum and Brachypodium. The closest flanking markers identified after QTL analysis on the previous marker saturated map (Fig. 1B), were used to select the target region for comparative analysis on both 7HS and 7HL intervals. Identification of syntenic regions at the three model species was performed basically according to Perovic et al. (2004). Nucleotide sequences of flanking markers were used as queries for BLASTN search (Altschul et al. 1997) against the *Oryza sativa* ssp. *japonica* IRGSP Build5 (http://rapdb.dna.affrc.go.jp), *Sorghum bicolor* release v1.0 (http://www.phytozome.net/sorghum.php) and *Brachypodium distachyon* JGI 8X (http://www.brachypodium.org) genomes. The sequences of all genes located at the syntenic region on each genome were then used in a BLASTN search to screen barley ESTs stored in the HarvEST database Assembly#35 (http://harvest.ucr.edu) using cut-off parameters of *E*-value$\leq e^{-10}$, identity $\geq$ 80% and a minimum of 100 bp match length. In the third step, barley ESTs that yielded a positive

hit to any of the three genomes were subjected to BLASTN against all available genomic sequences of rice, sorghum and Brachypodium. Only those barley ESTs that had their most significant hit (*E*-value $\leq e^{-10}$) with the rice, sorghum or Brachypodium chromosome identified in the first instance were considered as potential orthologous and employed in further analysis.

The consensus sequence from each barley unigene was downloaded from HarvEST and used as a template for design of specific primers. The location of primers in regions with a potentially high rate of polymorphisms (3´UTR, 5´UTR and introns) was emphasized by comparing the barley unigene sequence with the genomic sequence of the corresponding putative ortholog in rice, sorghum or Brachypodium using the online version of the software Spidey (http://www.ncbi.nlm.nih.gov/spidey). Primers for sequencing each barley unigene were designed using Primer 3 software with parameter setting of product size up to 400 bp. When the length of the unigene was around 1 kb, several primer combinations were designed to cover the whole sequence. Analysis of polymorphisms on PCR amplicons and development of new CAPS or pyrosequencing-based markers was performed as described above.

Linkage and QTL analysis

Ninety-four informative lines that showed recombination at the target intervals were used to map the new markers. The previously mapped microsatellite markers on 7H (Silvar et al. 2010b) were used as a framework to place the new markers. Genetic distances were calculated by minimizing the number of recombinants within the progeny. Linkage analyses were performed with JoinMap 4.0 (van Ooijen 2006), using Kosambi's map function and a minimum logarithm of the odds ratio (LOD score) of 3.

QTL analysis was performed using the Multiple QTL Model (MQM) (Jansen and Stam 1994) implemented in MapQTL 5.0 (van Ooijen 2004). Several rounds of analysis with cofactors were

conducted until a stable LOD profile was reached. The LOD threshold for QTL detection was calculated by permutation test with 1,000 iterations and a genome-wide significance level of 0.05.

Results

Marker saturation and QTL analysis

In a first step for map saturation, 94 lines with recombinations across the QTL regions were identified on both QTL intervals, out of the 262 original RIL population. Markers GBM1126 and GBM1060 on 7HS and markers GBM1102 and EBmac0755 on 7HL were designated as flanking markers for the QTL intervals (Fig. 1A, Silvar et al. 2010b). These four markers were used to anchor our population with three reference barley populations. Marker GBM1126 was polymorphic in the I×F population, GBM1060 showed polymorphism in S×M and D×R populations, GBM1102 was mapped on D×R, and EBmac0755 on S×M and D×R. This information together with the available data from Stein et al. (2007) served to select for GBS, GBR and GBM markers within the interesting intervals (Table S1). Marker WBE106, which is close to EBmac0755 in Marcel et al (2007), and marker GBM1006, proximal to GBM1102 (Varshney et al. 2007), were chosen based on their polymorphism in SBCC097 and Plaisant. Among GBS markers, only GBS0572 and GBS0028 turned out to be polymorphic between the parental lines. Ten GBR (RFLP) markers were converted to PCR-based markers (named as QBS) by designing specific primers for amplification and sequencing. Two of them (QBS8 and QBS12) did not amplify any PCR product. Among the other eight, five showed a SNP between the parents, 3 at the QTL on 7HS and 2 at the target region on 7HL (Table S1, Fig. 1B).

Forty-six lines showing recombination in one or both QTL regions on chromosome 7H, and unequivocal phenotyping, were selected for the Illumina genotyping assay. Out of 1,563-SNP BOPA1 markers, 160 were assigned to chromosome 7H. Among these, 64 showed polymorphism between SBCC097 and Plaisant. These new markers were combined with previous microsatellite

markers mapped on chromosome 7H (Silvar et al. 2010b) and a new map was constructed using only these 46 recombinant lines. Four and ten markers mapped within the target regions on 7HS and 7HL, respectively (data not shown). These 14 markers were selected to genotype the additional 48 RIL, in order to improve the quality of the map, using a newly developed pyrosequencing protocol (Silvar et al. 2011b). Out of these, marker 11_0080 amplified multiple bands and marker 11_1043 did not show the polymorphism previously reported by Close et al. (2009) and were discarded. Four markers (two within the target interval) showed linkage to markers on the 7HS region whereas eight mapped at the 7HL region (Table S1, Fig.1B).

Markers from Sato et al. (2009) were selected according to information available at HarvEST and Sato and Takeda (2009). Ninety-three percent of these markers are formatted in Affymetrix Barley 1 GeneChip (HarvEST Assembly#25). By comparing the unigenes on this assembly with the unigenes on which the BOPA1 markers were designed (Assembly#35), we could infer which markers were probably designed on the same unigenes from different assemblies. We selected the markers that were potentially located at the QTL intervals on chromosome 7H. For example, SNP marker 11_0619 is designed on unigene 2036 (Assembly#35) and it corresponds to unigene 5570 in Assembly#25 and, therefore, to marker k08205 from Sato et al. (2009). Some of these markers co-segregated in the population Haruna Nijo×H602, and thus were not selected. Twenty-one markers were chosen according to this procedure. Out of these, primers for marker k00065 did not generate any amplicon and primers for k06838 amplified multiple bands. The other nineteen selected markers were sequenced, SNPs were detected in eleven of them, four corresponding to the QTL interval on 7HS and seven markers to the QTL on 7HL (Table S1, Fig. 1B).

Polymorphism rate was quite high among all screened markers and the majority of sequences displayed more than one SNP between parental lines. Indeed, up to 10 SNPs between SBCC097 and Plaisant were identified in the sequence BU989280 (marker QBS9), with a length of ca. 1140bp.

In total, out of fifty-two previously published markers examined for this first saturation step, thirty-two were polymorphic and could be integrated into the previously published genetic map of SBCC097×Plaisant (Silvar et al. 2010b) (Fig. 1B). The current map for chromosome 7H comprises two linkage groups, rather than the three described earlier (Silvar et al. 2010b) (Fig. 1A and 1B). Each linkage group approximately corresponds to the short and long arm of chromosome 7H. Nine markers mapped at the GBM1126-GBM1060 interval on 7HS and 14 at the GBM1122-EBmac0755 interval on 7HL (Fig. 1B), whereas 9 markers were close, but not within, the QTL intervals. With this abundance of markers, it was not surprising to find four groups of co-segregating markers (Fig 1B).

QTL analysis performed with MapQTL on the saturated map, using phenotypic data obtained with four *B. graminis* isolates (Silvar et al. 2010b) markedly reduced the size of the QTL intervals (Fig 1C). The 7HS QTL was non-significant for isolate R178 but, for the other isolates, it was located in an interval of ca. 6.7 cM, between markers QBS3 and GBM1060 (Fig. 1C) (compared to 12.2 cM in the original work). The QTL on 7HL was detected for all four isolates and it now spans over a genetic distance of ca. 3 cM depending on the isolate (interval k02857-11_0115), with the peaks at 11_0934 for isolates R79, R126 and R180 or EBmac0755 for isolate R178 (Fig. 1C). In this case, the width of the original interval was not accurately assessed, since the flanking markers mapped at the extremes of two linkage groups, over a span of around 40 cM (Silvar et al. 2010b).

Comparative analysis on 7H QTL intervals

The new flanking markers of each QTL were used as starting points for a second step of marker enrichment, based on a comparative approach using rice, sorghum and Brachypodium genomes. BLASTN search using sequences of flanking markers of the 7HS QTL (QBS3-GBM1060) identified a syntenic region of 95 Kb on chromosome 6 (Os06) of rice (from gene Os06g0114000 to gene Os06g0116300), of 130 Kb on chromosome 10 (Sb10) of sorghum (from Sb10g001120 to

Sb10g001350) and of 203 Kb on chromosome 1 (Bd1) of Brachypodium (from gene Bradi1g50410 to gene Bradi1g50610). These regions include in total 63 putative orthologous genes, 20 for rice, 22 for sorghum and 21 for Brachypodium. Flanking markers of the 7HL QTL (k02857-11_0115) identified 23, 30 and 20 putative orthologs in rice, sorghum and Brachypodium, respectively, which covered a 198 Kb region in rice (from gene Os06g0651200 to Os06g0655100), a 276 Kb region in sorghum (from Sb10g025780 to Sb10g026070) and a 199 Kb region in Brachypodium (from Bradi1g31080 to Bradi1g31270). Curiously, the comparative genomic analysis between barley and Brachypodium revealed that the barley 7HS interval hits a syntenic region on the proximal part of Bd1L, whereas the interval k02857-11_0115 on barley 7HL showed homology to a region on the proximal part of Bd1S. The sequences of all 136 putative orthologous genes were employed to screen the HarvEST barley unigene database (Assembly#35). Two genes from rice (Os06g0652500 and Os06g0653300) were not considered due to the small size of the coding sequences. Four, thirteen and five genes of rice, sorghum and Brachypodium, respectively did not hit any barley unigene above an *E*-value of e^{-10}. Sixty-one and eighty barley unigenes were identified for the 7HS and 7HL intervals, respectively. All 141 barley unigenes were employed in a second step of BLASTN against the publicly available whole genome sequences of the three grass species. Only unigenes with the first hit to Os06 of rice, Sb10 of sorghum and Bd1 of Brachypodium were retained and considered as true orthologous loci. The other ESTs exhibiting the best match elsewhere in the reference genomes likely represent homologous or paralogs in each genome. At this point, 104 (73.7%) barley hits, 48 at 7HS and 56 at 7HL, were selected for further work. In the 7HS region, 25 out of 48 hits were common to the three genomes, 2 to rice and Brachypodium, 7 to sorghum and Brachypodium, and 2, 4 and 8 were specific for rice, sorghum and Brachypodium, respectively (Fig. 2A). Among the 56 hits for 7HL, 26 were common to the three genomes, and 3 to rice and sorghum, 4 to rice and Brachypodium and 8 shared common hits to sorghum and Brachypodium. Four barley unigenes were identified based only on homology to sorghum and

eleven barley unigenes were orthologous only to the Brachypodium genome (Fig. 2B). Notoriously, species specific genes, which possess a counterpart only in barley, were identified for rice (1.9%), sorghum (5.8%) and Brachypodium (11.6%). Information on selected barley unigenes and their homologies to the three reference genomes is provided in Table S2.

Among all hits we were able to infer the location of some of the markers mapped in the previous step, based on the information available at HarvEST and Sato et al. (2009). For example, markers k03115 and k08921 seem to be developed from unigenes U35_1490 and U35_1176 on different assemblies, whereas markers k02857, 11_0934 and 11_0115 seem to correspond to unigenes U35_24124, U35_17138 and U35_7579, respectively. Those target unigenes served as the starting points for the selection of fifty barley unigenes, whose orthologous genes are located in the rice, sorghum and Brachypodium syntenic regions. Barley unigenes matching the same orthologs were avoided (Table S2). Primers were designed for amplification and sequencing of selected barley unigenes (Table S3). Twenty-five out of fifty screened unigenes were polymorphic and genetically mapped yielding twenty-five new loci. Most markers (sixteen) were genotyped as CAPS and in nine cases the SNP was converted into a marker for pyrosequencing as described earlier (Silvar et al. 2011b). Twelve new unigene-based markers were mapped within the 7HS target interval; eleven were located at the 7HL interval and two markers (QBS34 and QBS54) did not map on chromosome 7H (Fig. 3). A comparison of colinearity among the syntenic blocks on the four species indicated that the interval k02857-11_0115 on 7HL was inverted in barley compared to rice, sorghum and Brachypodium genomes. Apart from this, all 23 newly developed markers were almost in perfect colinearity with their corresponding orthologous genes in rice, sorghum and Brachypodium, although some small rearrangements affecting single genes were detected, for example for marker QBS58 (Fig. 3). Interestingly, markers designed on barley unigenes (QBS15, QBS17, QBS24, QBS25, QBS30, QBS36, QBS46, QBS52 and QBS61) with no counterparts in all

three reference genomes, but just in one or two, were mapped following the expected order according to their putative orthologs in the corresponding genome.

The addition of 23 new markers allowed the definition of narrower QTL intervals. To achieve this, the RILs were grouped according to the presence/absence of each resistance region. Then, only the lines that presented at most one QTL were selected. Five lines had to be discarded due to an apparent mismatch between marker information and disease scores. The remaining thirty-five informative lines are presented in Fig. 4. An examination of the twenty-three RILs lacking the 7HS resistance suggested the presence of the QTL co-segregating with marker QBS46 in 7HL (based on recombinations in two lines). A more conservative assessment, based on seven recombinants, placed the QTL in the interval k00557-QBS36 (Fig. 4). Among twenty lines without the resistance allele in the 7HL QTL, three lines define the most likely region conferring resistance to powdery mildew in 7HS, between markers QBS23 and Bmag206. Again, a conservative assessment, based on five recombinants, defines a wider interval, QBS3-Bmag206 (Fig. 4).

The protein homologies of all barley unigenes identified within the chromosomal regions harbouring each QTL were inspected using BLASTX search in the NCBI database. Six out of sixteen inspected sequences contained coding regions for proteins with predicted functions. The remaining ten are referred to as "hypothetical proteins". Among these, nine barley unigenes showed sequence similarity to the protein coded by their counterpart in rice or sorghum (Table 1).

Discussion

Marker enrichment of QTL regions

Marker saturation of chromosomal regions affecting a specific trait represents one of the first steps towards map-based gene isolation, but it also has the direct benefit of improving the precision during marker assisted selection. In a previous work, the regions conferring resistance to powdery mildew on the Spanish line SBCC097 were mapped to large confidence intervals on the short and

long arms of chromosome 7H (Silvar et al. 2010b). Increasing marker density within both QTL intervals is essential before further efforts towards either QTL pyramiding or gene cloning can be carried out.

In the present work, we first took advantage of all the available information on recently developed EST-derived markers (Stein et al. 2007; Sato et al. 2009; Close et al. 2009). The main advantage of these so-called functional markers is that, as long as there is an association between their molecular polymorphism and the relevant phenotypic trait, they can be postulated as candidate genes (Aubert et al. 2006). Additionally, EST-derived markers are specially suited for fine mapping based on comparative genomic analysis since sequence conservation between species is mainly restricted to coding regions (Tang et al. 2008). The choice of markers from barley resources was restricted to those derived from single mapping populations (marker position based on segregation data), to avoid potential variations in marker order derived from integrated (consensus) maps.

Some QBS markers were developed from barley ESTs-RFLP described by Stein et al (2007). RFLP are very robust markers, but their analysis is expensive and laborious. Here, we converted GBR markers into cheaper and easy-to-use PCR-based markers genotyped as cleaved amplified polymorphic sites (CAPS) or pyrosequencing makers. These new PCR markers proved to be locus-specific and showed mapping locations corresponding to their counterpart RFLP markers.

Sato et al. (2009) developed a high-density transcript linkage map with more than 3,000 3'EST-PCR based markers using a single double haploid (DH) population, i.e. Haruna Nijo×H602. Later on, Sato and Takeda (2009) integrated the BOPA1 markers with EST-markers from Sato et al. (2009). Due to the absence of common polymorphic markers between the SBCC097×Plaisant and Haruna Nijo×H602 populations at the target regions, we had to follow a different strategy to find Sato´s markers that might be within the QTL intervals. The BOPA1 markers coming from Illumina data and information available at HarvEST provided the clue for cross-referring to the map from Sato et al (2009), allowing the identification of synonymous markers targeting the same unigene.

For example, the marker GBM1060 comes from barley clone HW05A06u which is the unigene #1176 in assembly #35, but unigene #3326 in assembly #25. Marker k08921 (clone bah42j17) is represented in unigene #3326 in assembly #25. Both markers share the same hit to the UniProt database (UniRef90_Q84UD4) and they should have a similar position in SBCC097×Plaisant population, as it was demonstrated. Both Sato´s and OPA-SNP resources provide a large and complementary source of molecular markers for saturation purposes. Addition of all new markers allowed to delimit the QTL regions to smaller intervals compared with previously published data (Silvar et al. 2010b) and to define closer flanking markers for further fine mapping.

Syntenic integration of QTL conferring resistance to powdery mildew

Since the release of the sequenced rice genome (Goff et al. 2002; Yu et al. 2002), several fine mapping strategies for different *Triticeae* species took advantage of synteny-based approaches (Perovic et al. 2004; Liu et al. 2006; Drader et al. 2009; Mayer et al 2009). Recently, the whole genome sequencing of other grass species, such as *S. bicolor* (Paterson et al. 2009) and *B. distachyon* (International Brachypodium Initiative 2010), has shed light on the diversification and phylogenetic relationships among grasses. These new reference genomes represent additional tools for syntenic integration and facilitate the genetic studies of larger cereal genomes (Higgins et al. 2010; Xue et al. 2010).

In the present study, we investigated the potential of all these genomic tools to develop additional genetic markers within target genomic regions by exploiting the colinearity between cereal and grass reference genomes. The success of this approach highly depends on the strategy employed for the *in silico* selection of the synteny-based regions and candidate ESTs. In this work, we followed a "reciprocal BLASTN search" procedure similar to that proposed by Perovic et al. (2004). According to this method, the putative orthologous genes were only confirmed after two steps of BLASTN searches, which allowed for effective removal of putative paralogous and homologous.

Comparative genomic analysis identified syntenic regions in Os06 of rice, Sb10 of sorghum and Bd1 of Brachypodium. Previous studies highlighted a syntenic relationship between barley chromosome 7H and rice chromosome 6 (Devos 2005; Stein et al. 2007; Thiel et al. 2009). However, the syntenic regions to Brachypodium and sorghum had not been clearly reported. Notoriously, genomic comparisons reveal reorganization in conserved regions of Brachypodium Bd1 when compared to the other species. Thus, barley 7HS and 7HL target regions identified inverted syntenic blocks on Bd1. This result confirms the grass chromosome evolution model (Salse et al. 2008; Bolot et al. 2009; International Brachypodium Initiative 2010), which hypothesized that syntenic blocks on distal parts of barley chromosome 7H and centromeric region of Brachypodium Bd1 derived from a common ancestral chromosome that suffered multiple rearrangements to shape the actual cereal and grass chromosome structures.

The largest syntenic regions for the resistance mapped on barley chromosome 7H were identified in sorghum (406 Kb) and Brachypodium (402 Kb). The gene content of these two intervals was higher in sorghum, harbouring 52 genes in total. However, only 75% of them presented hits on barley unigenes. This absence of hits may be explained by different factors, such as the time of divergence among species and the speed of divergence at certain loci, but may also be affected by the quality of chromosome annotation of model species. After the last step of BLASTN comparison, the higher number of barley unigenes, corresponding to “true” orthologous, were identified through the Brachypodium syntenic region (Fig. 2). The slightly better performance of this model genome in order to identify barley unigenes from putative orthologous may be explained by the closer coancestry between Brachypodium and barley (Bossolini et al. 2007; Chen et al. 2009; Drader and Kleinhofs 2010).

Fifty barley unigenes, identified according to the synteny-based approach, were selected for marker development. Of the 60 primer pairs tested for polymorphism between SBCC097 and Plaisant, 25 pairs (41.6%) amplified polymorphic fragments on different unigenes. Such polymorphism rate was

similar to the 40% observed on chromosome 7H after genotyping with SNP-BOPA markers, which are mainly designed on exons (Close et al. 2009). We expected that pointing at 3' and 5'UTR regions and introns for amplification would increase the chances of polymorphism in barley unigenes, according to previous reports on soybean (Van et al. 2005), which detected higher SNP rates in non-coding regions than in exons. Twenty-three out of twenty-five polymorphic markers (92%) mapped within the defined target intervals. This percentage is very high in comparison to previous studies on comparative genomics of cereals (Mammadov et al. 2005; Drader et al. 2009; Liu et al. 2010) and demonstrates the good level of conservation of sequence order between these species, as well as the suitability of our "reciprocal BLASTN" procedure to correctly identify the orthologous genes.

The level of colinearity between barley and the other three reference genomes is nearly identical to the colinearity among rice, sorghum and Brachypodium, thus suggesting an evolutionary conservation of the genes located in these regions among the four species. One remarkable aspect was the presence of a rearrangement of the targeted resistance region on 7HL, resulting in an inversion between barley and the three grass genomes. Marker k02857 is distal to marker 11_0115 on the barley genetic map. However, the rice, sorghum and Brachypodium orthologous genes of those two markers are in opposite order in their respective physical maps. The positions of other markers between these two also support the presence of an inversion. A similar inversion on the long arm of chromosome 7H was proposed by Stein et al. (2007) after comparing a consensus transcript map of barley with the rice chromosomes, and it can be visualized on HarvEST v1.77 using the tool "Barley Integrated Map" (http://harvest.ucr.edu/). The fact that the inversion was only detected in barley suggests that this rearrangement on 7HL occurred after the most recent divergence event of this species in the phylogenetic tree of the grasses. Small inversions were also observed in the order of some syntenic genes in grasses. For example, the syntenic region on Bd1 between markers U35_18765 and U35_40903 appears inverted related to the physical map of

sorghum and rice and the genetic map of barley. In general, rearrangements in syntenic regions across species appear to be a common attribute (Devos 2005; Pourkheirandish et al. 2007; Chen et al. 2009).

Another interesting feature is that each reference genome contained a few species specific genes which are absent from at least one of the model species addressed, but are conserved in the barley genome. For example, genes Os06g0115500, Sb10g001330 and Bradi1g31190 seem to be exclusively present in rice, sorghum and Brachypodium, respectively, and they conserved their counterpart only in barley, even maintaining the gene order. Although purely speculative, these genes may be considered as "orphans", i.e., genes in which the corresponding orthologs has either been lost or has failed to be predicted in some lineages (Guo et al. 2007; Merkeev and Mironov 2008).

Several comparative genomic studies including rice, Brachypodium, sorghum and a member of the *Triticeae* pointed out a better synteny between Brachypodium and the *Triticeae* than between rice or sorghum and the *Triticeae* (Opanowicz et al. 2008; Faricelli et al. 2009). Looking at our results, we cannot clearly conclude that any of the three grasses used was outperforming the others in its quality as a model for barley, at least in the regions targeted. On the contrary, we must highlight the good performance of all three genomes, which provide common and individual blocks of genes that can be assembled like building blocks to reconstitute the large genome of related barley. Therefore, they should be considered together as an integrated resource for fine mapping and gene identification.

The addition of new synteny-based markers resulted in an average density of 1 marker per 0.6-0.7 cM within the previously reported QTL regions, narrowing down the resistance regions to much smaller intervals. Inspection of all predicted proteins located in the shortened QTL regions did not reveal the presence of resistance gene candidates such as the "nucleotide binding site-leucine rich repeat" (NBS-LRR) proteins or "serine/threonine-protein kinases", the two major classes of

resistance genes. The majority of predicted proteins are referred as “hypotethical protein”, meaning that they have hits to uncharacterised proteins without predicted functions. In any case, identification of candidate genes would be beyond the genetic resolution of this work.

Successful fine mapping of single genes and quantitative trait loci by extensive exploitation of the synteny among genomes has been widely reported (Pourkheirandish et al. 2007; Hanemann et al. 2009; Liu et al. 2010; Xue et al. 2010). However, strategies relying on the micro-colinearity of related genomes have sometimes proven fruitless in fine mapping and candidate gene identification. (Tarchini et al. 2000; Bulgarelli et al. 2004; Pourkheirandish et al. 2007). The low genetic resolution of the SBCC097×Plaisant population does not clarify the status of micro-colinearity at the targeted regions. However, the newly developed synteny-based markers should enable the effective screening for recombinants of large F_2 populations, which also will allow validating and resolving the order of tightly linked markers as well as the further search for candidate genes based on micro-colinearity of related genomes. Similarly, those markers will be extremely useful in barley breeding programs aimed at introgression of the novel powdery mildew resistance found in the Spanish line SBCC097 via marker-assisted selection approaches.

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Table 1 Putative function of those barley unigenes mapped at the QTL regions deduced by BLASTX sequence comparison against the NCBI database

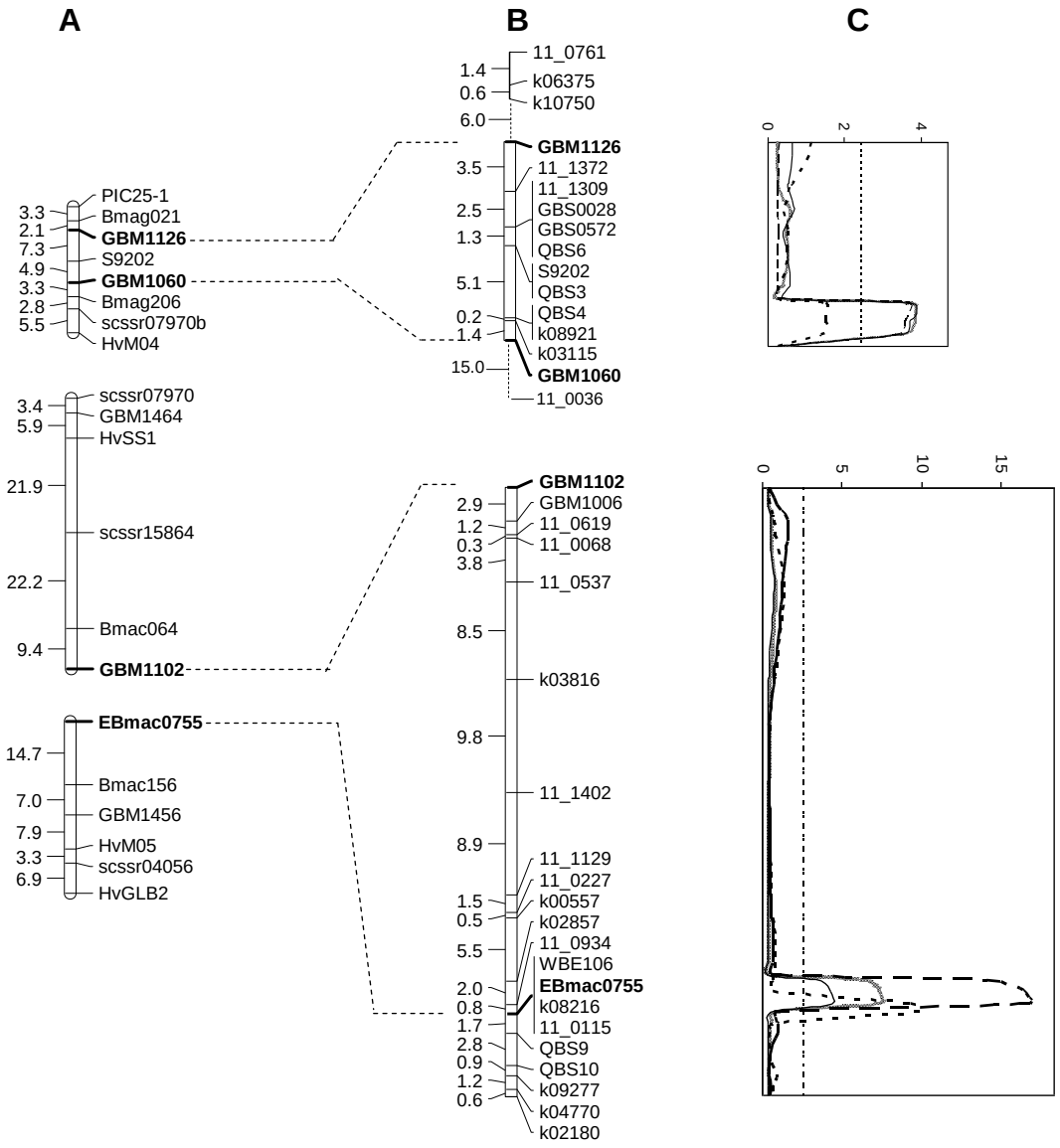
Marker	Unigene	Crom	Protein homology	*E* -value
QBS28	U35_5413	7HS	Hypotetical protein similar to Sb10g001330	$3e^{-76}$
QBS24	U35_43149		Hypothetical protein similar to Os06g0115800	$2e^{-24}$
k03115	U35_1490		Hypothetical protein	$1e^{-173}$
QBS25	U35_1177		GAMYB-binding protein	$9e^{-71}$
k08921	U35_1176		GAMYB-binding protein	0
QBS29	U35_25087		Transcription activator GRF3	$9e^{-71}$
QBS30	U35_6111		Hypothetical protein simmilar to Os06g0116300 (putative syntaxin)	$1e^{-144}$
GBM1060	-		GAMYB-binding protein	$7e^{-15}$
QBS61	U35_3292	7HL	CONSTANS-like protein	$2e^{-150}$
QBS52	U35_23045		Hypothetical protein similar to Os06g0654000	$3e^{-19}$
QBS50	U35_7866		Hypothetical protein similar to Os06g0654000 and Sb10g026010	$1e^{-90}$
QBS46	U35_49745		Protein transport protein SEC61 gamma subunit	$1e^{-29}$
QBS45	U35_38716		Hypothetical protein similar to Os06g0653800 and Sb10g025990	$7e^{-33}$
QBS44	U35_4068		Hypothetical protein similar to Os06g0653800 and Sb10g025990	0
11_0934	U35_17138		Hypothetical protein similar to Sb10g025930	$5e^{-88}$
QBS42	U35_11617		Hypothetical protein similar to Os06g0653000 and Sb10g025920	$2e^{-61}$

Fig. 1 Genetic linkage map of barley chromosome 7H developed in the SBCC097×Plaisant population before (A, Sivar et al. 2010b) and after (B) map saturation. (C) MQM LOD scans for resistance scores to different isolates of *B. graminis*. For comparison purposes, the two genetic maps are not drawn to scale. Flanking markers for both QTLs are shown **in bold** and connected with thin discontinuous lines

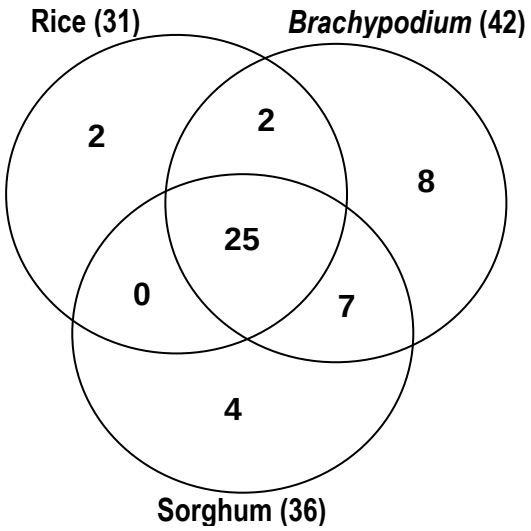
Fig. 2 Venn diagrams showing the number of common and single barley unigenes selected on chromosome 7HS (A) and 7HL (B) by using BLASTN against the publicly available sequences of rice, sorghum and Brachypodium. Numbers in brackets indicate the number of barley unigenes identified by each reference genome

Fig. 3 Comparative maps of syntenic regions of barley chromosome 7H in relation to sorghum chromosome 10 (Sb10), rice chromosome 6 (Os06) and Brachypodium chromosome 1 (Bd1). Barley flanking markers employed as starting point for the comparative approach are **in bold**, markers developed on barley unigenes that showed only one hit to one reference genome are *underlined*, and markers developed on barley unigenes that showed two matches to two reference genomes are *in italics*. *Asterisks* (*) indicate barley unigenes that have not been mapped but show high similarity to markers previously included in the enriched map (see Fig. 1B). Note that physical maps of sorghum, rice and Brahcypodium are not on the same scale as the genetic map of barley. Note that both arms of Bd1 are shown on inverted position. For the sake of clarity, direct comparison between sorghum and barley are not indicated, but the homology to the sorghum genome is represented through the rice orthologous (for better clarification see Table S2)

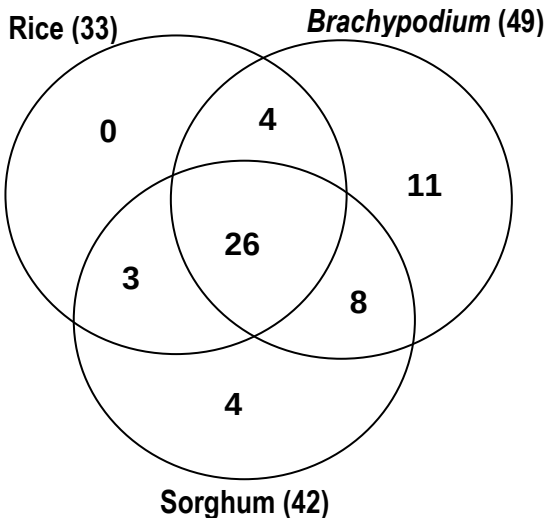
Fig. 4 Diagram of the short and long arms of chromosome 7H in RILs of the SBCC097× Plaisant population showing recombination on the regions harboring QTL. Allelic identities are shown in *grey* for SBCC097 and *white* for Plaisant. For each RIL, the disease score (ranging from 0 to 4) is presented for each isolate and for the mean of four isolates. Potential chromosomal regions conferring resistance to *B. graminis* are highlighted in *grey* and *light grey*. For the sake of clarity, the interval k03816–11_0115 is represented for chromosome arm 7HL. *S* indicates susceptibility and *R* resistance

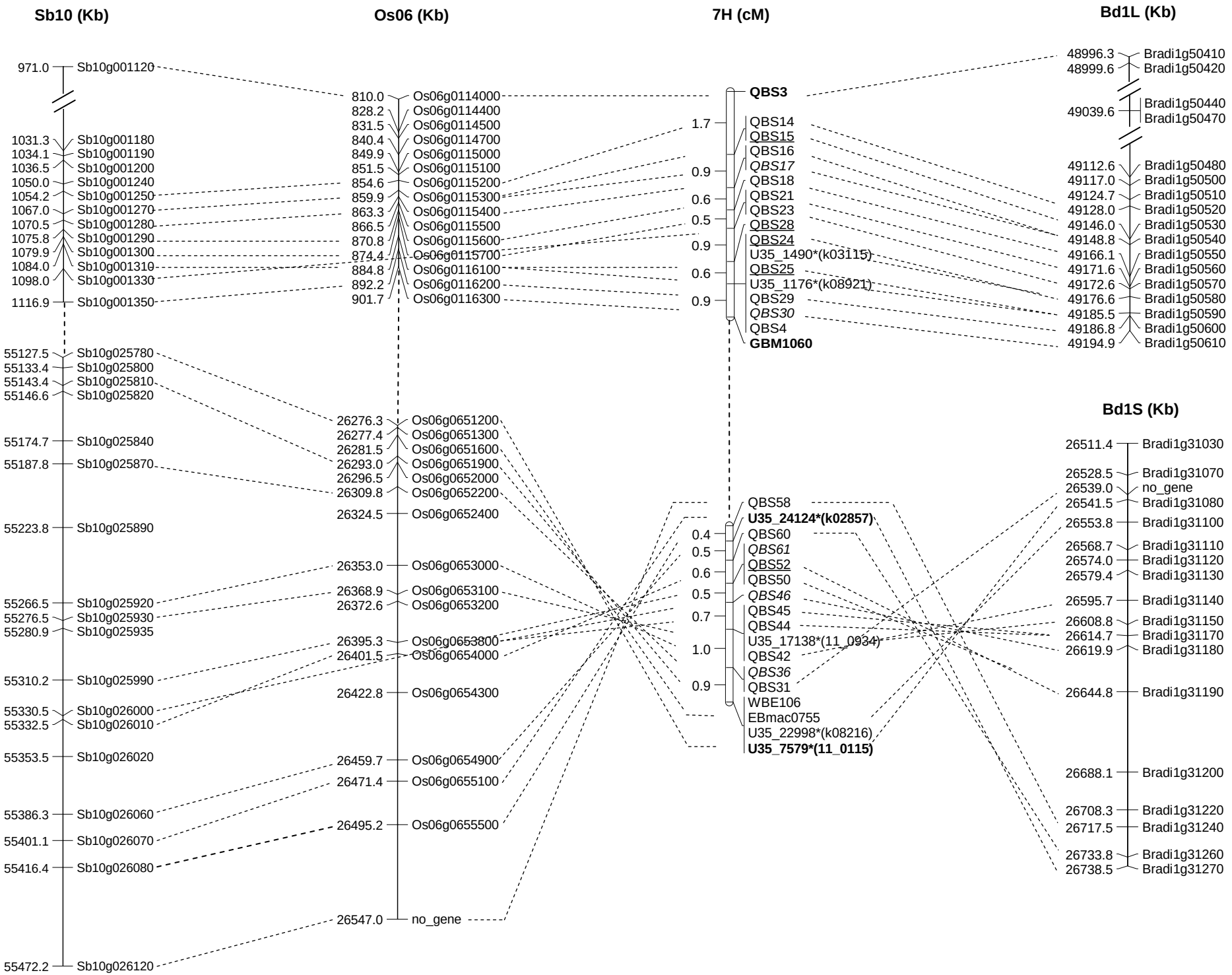

A
B
C
PIC25-1
Bmag021
GBM1126
S9202
GBM1060
Bmag206
scssr07970b
HvM04
3.3
2.1
7.3
4.9
3.3
2.8
5.5
11_0761
k06375
k10750
1.4
0.6
6.0
GBM1126
11_1372
11_1309
GBS0028
GBS0572
QBS6
S9202
QBS3
QBS4
k08921
k03115
GBM1060
11_0036
3.5
2.5
1.3
5.1
0.2
1.4
15.0
0
2
4
scssr07970
GBM1464
HvSS1
scssr15864
Bmac064
GBM1102
3.4
5.9
21.9
22.2
9.4
GBM1102
GBM1006
11_0619
11_0068
11_0537
k03816
11_1402
11_1129
11_0227
k00557
k02857
11_0934
WBE106
EBmac0755
k08216
11_0115
QBS9
QBS10
k09277
k04770
k02180
2.9
1.2
0.3
3.8
8.5
9.8
8.9
1.5
0.5
5.5
2.0
0.8
1.7
2.8
0.9
1.2
0.6
0
5
10
15
EBmac0755
Bmac156
GBM1456
HvM05
scssr04056
HvGLB2
14.7
7.0
7.9
3.3
6.9

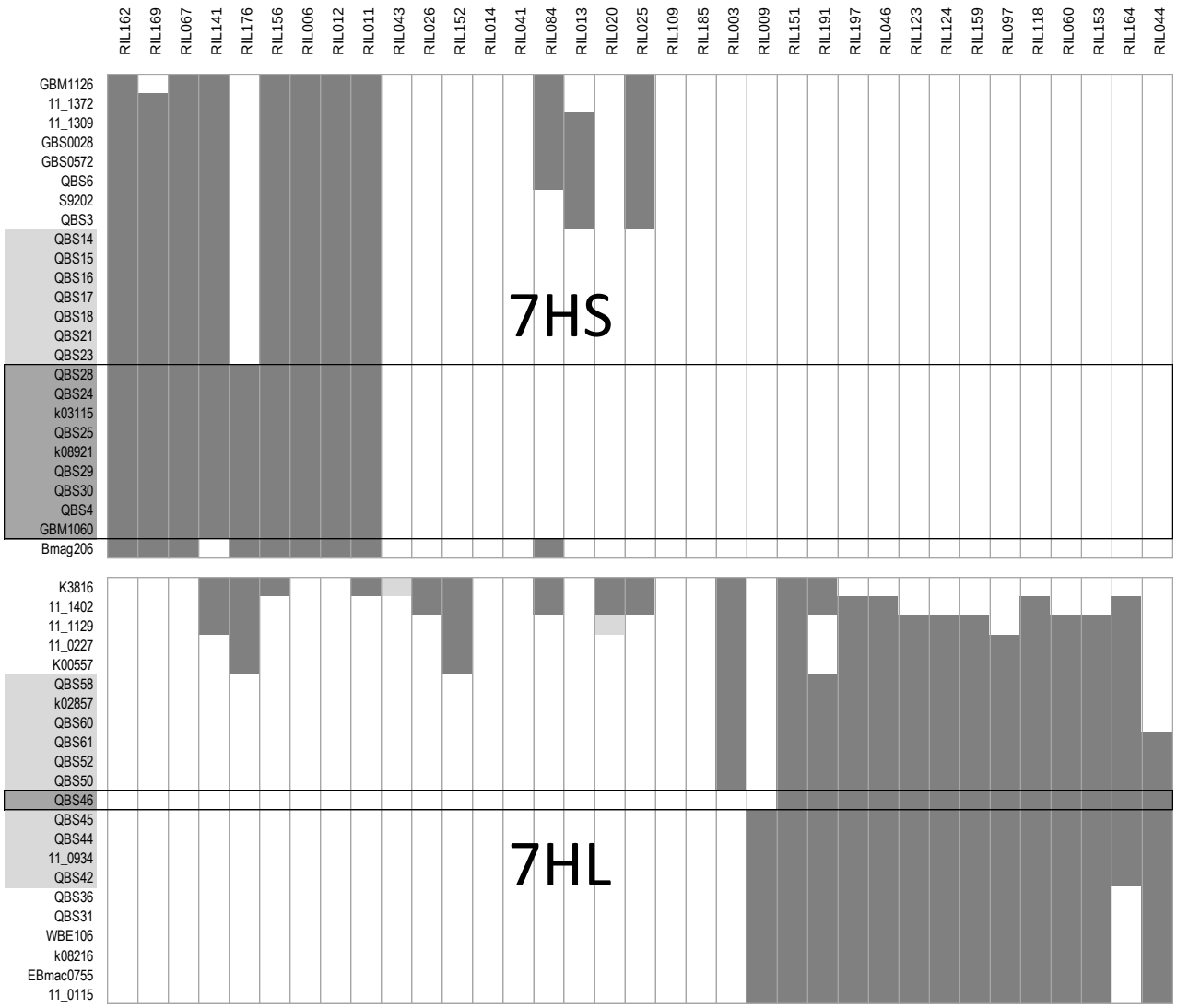
A


Rice (31)
Brachypodium (42)
2
2
8
25
0
7
4
Sorghum (36)

B


Rice (33)
Brachypodium (49)
0
4
11
26
3
8
4
Sorghum (42)


Sb10 (Kb)
Os06 (Kb)
7H (cM)
Bd1L (Kb)
Bd1S (Kb)
971.0 Sb10g001120
1031.3 Sb10g001180
1034.1 Sb10g001190
1036.5 Sb10g001200
1050.0 Sb10g001240
1054.2 Sb10g001250
1067.0 Sb10g001270
1070.5 Sb10g001280
1075.8 Sb10g001290
1079.9 Sb10g001300
1084.0 Sb10g001310
1098.0 Sb10g001330
1116.9 Sb10g001350
55127.5 Sb10g025780
55133.4 Sb10g025800
55143.4 Sb10g025810
55146.6 Sb10g025820
55174.7 Sb10g025840
55187.8 Sb10g025870
55223.8 Sb10g025890
55266.5 Sb10g025920
55276.5 Sb10g025930
55280.9 Sb10g025935
55310.2 Sb10g025990
55330.5 Sb10g026000
55332.5 Sb10g026010
55353.5 Sb10g026020
55386.3 Sb10g026060
55401.1 Sb10g026070
55416.4 Sb10g026080
55472.2 Sb10g026120
810.0 Os06g0114000
828.2 Os06g0114400
831.5 Os06g0114500
840.4 Os06g0114700
849.9 Os06g0115000
851.5 Os06g0115100
854.6 Os06g0115200
859.9 Os06g0115300
863.3 Os06g0115400
866.5 Os06g0115500
870.8 Os06g0115600
874.4 Os06g0115700
884.8 Os06g0116100
892.2 Os06g0116200
901.7 Os06g0116300
26276.3 Os06g0651200
26277.4 Os06g0651300
26281.5 Os06g0651600
26293.0 Os06g0651900
26296.5 Os06g0652000
26309.8 Os06g0652200
26324.5 Os06g0652400
26353.0 Os06g0653000
26368.9 Os06g0653100
26372.6 Os06g0653200
26395.3 Os06g0653800
26401.5 Os06g0654000
26422.8 Os06g0654300
26459.7 Os06g0654900
26471.4 Os06g0655100
26495.2 Os06g0655500
26547.0 no_gene
1.7
0.9
0.6
0.5
0.9
0.6
0.9
0.4
0.5
0.6
0.5
0.7
1.0
0.9
QBS3
QBS14
QBS15
QBS16
QBS17
QBS18
QBS21
QBS23
QBS28
QBS24
U35_1490*(k03115)
QBS25
U35_1176*(k08921)
QBS29
QBS30
QBS4
GBM1060
QBS58
U35_24124*(k02857)
QBS60
QBS61
QBS52
QBS50
QBS46
QBS45
QBS44
U35_17138*(11_0934)
QBS42
QBS36
QBS31
WBE106
EBmac0755
U35_22998*(k08216)
U35_7579*(11_0115)
48996.3 Bradi1g50410
48999.6 Bradi1g50420
49039.6 Bradi1g50440
Bradi1g50470
49112.6 Bradi1g50480
49117.0 Bradi1g50500
49124.7 Bradi1g50510
49128.0 Bradi1g50520
49146.0 Bradi1g50530
49148.8 Bradi1g50540
49166.1 Bradi1g50550
49171.6 Bradi1g50560
49172.6 Bradi1g50570
49176.6 Bradi1g50580
49185.5 Bradi1g50590
49186.8 Bradi1g50600
49194.9 Bradi1g50610
26511.4 Bradi1g31030
26528.5 Bradi1g31070
26539.0 no_gene
26541.5 Bradi1g31080
26553.8 Bradi1g31100
26568.7 Bradi1g31110
26574.0 Bradi1g31120
26579.4 Bradi1g31130
26595.7 Bradi1g31140
26608.8 Bradi1g31150
26614.7 Bradi1g31170
26619.9 Bradi1g31180
26644.8 Bradi1g31190
26688.1 Bradi1g31200
26708.3 Bradi1g31220
26717.5 Bradi1g31240
26733.8 Bradi1g31260
26738.5 Bradi1g31270



	R	R	R	R	R	R	R	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S	R	R	R	R	R	R	R	R	R	R	R	R	R
Isolate R79	1	1	1	1	2	1-2	1	1	1	2-3	2-3	2-3	2-3	2-3	2-3	2-3	3	2-3	3	3	2	2-3	1	1	1	1	1	1-2	1-2	1-2	1-2	1-2	1-2	1-2	1-2
Isolate R126	0	0	1	1	1	1	1	1	1	2-3	2-3	2-3	2-3	3	2-3	2-3	2	2-3	2-3	2	2	1-2	0	0	0	0	0	0	0	0	0	1	1	1	1
Isolate R178	-	0	1	1-2	0	1	1-2	1-2	2	2-3	2-3	2-3	2-3	2	2-3	2-3	2-3	3-4	2-3	2-3	2-3	2-3	0	0	0	0	0	1	1	1	1	1	1	1	1-2
Isolate R180	0	1	1	1	1-2	1	1	1	1	2	2-3	2-3	2-3	2-3	2-3	3	3-4	2-3	3	3-4	2	2-3	1	1	1	1	1-2	1-2	1-2	2	2	1-2	1-2	1-2	1-2
Average	0.33	0.5	1	1.13	1.13	1.13	1.13	1.13	1.25	2.38	2.5	2.5	2.5	2.5	2.5	2.63	2.75	2.75	2.75	2.75	2.13	2.25	0.5	0.5	0.5	0.5	0.63	1	1	1.13	1.13	1.25	1.25	1.25	1.38