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# A high-density consensus map of barley to compare the distribution of QTLs for partial resistance to *Puccinia hordei* and of defence gene homologues

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**Abstract** A consensus map of barley was constructed based on three reference doubled haploid (DH) populations and three recombinant inbred line (RIL) populations. Several sets of microsatellites were used as bridge markers in the integration of those populations previously genotyped with RFLP or with AFLP markers. Another set of 61 genic microsatellites was mapped for the first time using a newly developed fluorescent labelling strategy, referred to as A/T labelling. The final map contains 3,258 markers spanning 1,081 centiMorgans (cM) with an average distance between two adjacent loci of 0.33 cM. This is the highest density of markers reported for a barley genetic map to date. The consensus map was divided into 210 BINs of about 5 cM each in which were placed 19 quantitative trait loci (QTL) contributing to the partial resistance to barley leaf rust (*Puccinia hordei* Otth) in five of the integrated populations. Each parental barley combination segregated for different sets of QTLs, with only few QTLs shared by any pair of cultivars. Defence gene homologues (DGH) were identified by tBlastx homology to known genes involved in the defence of plants against microbial pathogens. Sixty-three DGHs were located into the 210 BINs in order to identify candidate genes responsible for the QTL effects. Eight BINs were co-occupied by a QTL and DGH(s). The positional candidates identified are receptor-like kinase, *WIR1* homologues and several defence response genes like peroxidases, superoxide dismutase and thaumatin.

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## Introduction

Linkage maps are essential tools in identifying genes responsible for polymorphic traits like disease resistance versus susceptibility, for comparing the genomes of different species, for map-based gene isolation and for genome sequencing. The earliest type of molecular markers used to construct genetic linkage maps were restriction fragment length polymorphisms (RFLP), which were applied in barley to genotype the Igri × Franka and the Steptoe × Morex populations (Graner et al. 1991; Kleinhofs et al. 1993). Nowadays, RFLPs have largely been replaced by different types of PCR-based molecular markers such as amplified fragment length polymorphisms (AFLP), single nucleotide polymorphisms (SNP) and single sequence repeats (SSR). The AFLP technology was used in barley to genotype the Oregon Wolfe Barley, L94 × Vada, SusPtrit × Vada and SusPtrit × Cebada Capa populations (Costa et al. 2001; Qi et al. 1998a; Jafary et al.

2006a, b). The so-called expressed sequence tags (EST) are one of the most informative sources of genetic markers because they represent partial sequences of genes and hence, those markers should map at the position of the corresponding gene. The RFLP, SNP and SSR technologies are actually used to saturate the barley genome with EST-based markers (Thiel et al. 2003: SSR; Sato et al. 2004: SNP; Varshney et al. 2004, 2006a: RFLP, SNP, SSR; Rostoks et al. 2005: SNP, SSR). Then, one of the greatest challenges is the integration of these different maps, genotyped by several groups using different techniques and different mapping populations, to produce a unified picture of the barley genome. In the past, two consensus maps based on RFLP markers (Langridge et al. 1995; Qi et al. 1996), containing 587 and 880 markers, respectively, and one consensus map combining 700 RFLP, AFLP and SSR markers (Karakousis et al. 2003) were constructed for barley.

The most important use for linkage maps is to identify chromosomal locations containing genes and quantitative trait loci (QTL) associated with traits of interest. QTL analysis provides a means to map several loci and to determine their interactions in a segregating cross (Borevitz and Chory 2004). Understanding the response of QTLs in different environments or genetic backgrounds can lead to the development of improved crop varieties through marker-assisted selection. If the genes underlying the QTL are known (i.e. the QTL has been "cloned"), then transgenic approaches can also be used to directly introduce beneficial alleles across intra- or inter-species boundaries (Borevitz 2004). Nevertheless, although map-based positional cloning has been used to isolate a large number of genes that inherit according to Mendelian ratios, such cloning is considered problematic for QTLs since genotypes cannot be unambiguously recognised from phenotypes of individual plants (Remington et al. 2001). Notably, this is the case for QTLs involved in disease resistance. An alternative approach to positional cloning of those QTLs is the candidate gene approach. The most common way to identify a candidate gene that may affect the QTL for resistance directly is to look for map co-segregation between genes of interest and QTLs for resistance (Pflieger et al. 2001). This approach has been applied in several experiments for different plant–pathogen systems (Faris et al. 1999; Wang et al. 2001; Trognitz et al. 2002; Ramalingam et al. 2003; Lanaud et al. 2004; Liu et al. 2004). However, in the end, it always remains to be determined whether the candidate gene and the QTL map on the same position on the linkage map by chance or indeed because the candidate gene really is responsible for the phenotype

determined by the QTL. The process of identifying candidate genes relies on the available information gained through the mapping of QTLs and of gene sequences with known function. Since biological functions are attributed to an increasing number of gene sequences, keeping gene annotations up to date with current publications is an important task.

In this paper, we report the merge of the available linkage mapping data of six different barley populations with mapped QTLs for partial resistance to barley leaf rust (*Puccinia hordei* Otth) and defence gene homologues.

## Materials and methods

### Plant material (mapping populations)

Three recombinant inbred line (RIL) populations and three doubled haploid (DH) populations were used to construct a consensus map of barley. The RIL populations have been developed at Wageningen University (Wageningen, The Netherlands), and consist of lines derived from crosses between L94 and Vada (L × V, 103 lines; Qi et al. 1998a), between SusPtrit and Vada (Su × V, 152 lines; Jafary et al. 2006a), and between SusPtrit and Cebada Capa (Su × CC, 113 lines; Jafary et al. 2006b). The two DH populations consisting of lines derived from crosses between Steptoe and Morex (St × M, 150 lines; Kleinhofs et al. 1993) and between *Dom* and *Rec* (OWBs, 94 lines; Costa et al. 2001), have been developed in North America and are reference mapping populations subject to extensive genotyping and phenotyping. The third DH population consists of lines derived from a cross between Igri and Franka (I × F, 71 lines; Graner et al. 1991), which were used to construct the first complete RFLP linkage map of barley.

### Available linkage mapping data

The available data sets of the three RIL populations consisted predominantly of AFLP markers (Table 1). For L × V, the segregation data of 568 markers were obtained from Qi et al. (1998a). For Su × V and Su × CC, the segregation data of 450 markers and of 506 markers, respectively, were obtained from Jafary et al. (2006a, b). The segregation data sets of the OWBs were downloaded from the Oregon State University (OSU) Barley Project web site (http://www.barleyworld.org/). Most of the 769 markers downloaded for the OWB population are AFLP markers (Table 1). The segregation data sets of the St × M and I × F populations were downloaded from the publicly

**Table 1.** Characteristics of the six barley populations used to construct the consensus map and numbers of marker loci and defence gene homologues (DGH) placed on the consensus map per molecular marker type

| Barley populations | | | Number of markers within populations | | | | | | | DGH no.[c] |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Name | Type | Lines | RAPD | RFLP | AFLP | SSR | Gene[a] | Other[b] | Total | |
| L × V | RIL | 103 | 0 | 0 | 785 | 138 | 5 | 29 | 957 | 11 |
| Su × V | RIL | 152 | 0 | 0 | 420 | 24 | 2 | 4 | 450 | 4 |
| Su × CC | RIL | 113 | 0 | 0 | 481 | 14 | 0 | 0 | 495 | 0 |
| OWBs | DH | 94 | 5 | 103 | 594 | 76 | 14 | 5 | 797 | 23 |
| St × M | DH | 150 | 11 | 421 | 0 | 177 | 11 | 15 | 635 | 21 |
| I × F | DH | 71 | 0 | 476 | 0 | 74 | 7 | 0 | 557 | 8 |

[a] The class "gene" comprises isozyme and morphological markers, and major disease resistance genes

[b] The class "other" comprises simple PCR markers such as SNP, SCAR and CAPS

[c] Number of defence gene homologues mapped in each population

available GrainGenes 2.0 databank (http://www.wheat. pw.usda.gov/). Those two data sets comprised 588 and 550 markers, respectively, and consisted predominantly of RFLP markers (Table 1).

Genetic mapping of PCR markers

DNA extraction was done according to the CTAB-based protocol of Steward and Via (1993), adjusted for 96-well format.

We scored an additional 235 AFLP markers segregating in L × V with 11 *Pst*I/*Mse*I primer combinations. The AFLP procedure was essentially performed as described by Vos et al. (1995) with some modifications according to Qi and Lindhout (1997). The selective *Pst* primer was labelled with IRD700 or IRD800 and the AFLP fingerprints generated on a LICOR 4200 DNA sequencer (LI-COR® Biosciences, Lincoln, NE, USA). The following primer combinations were run: P14M50, P14M54, P14M56, P14M61, P15M47, P15M51, P15M52, P15M53, P16M50, P16M51 and P17M47, following the nomenclature proposed by Qi and Lindhout (1997) and Bai et al. (2003). Additional primers were M52 (M00 + CCC), M53 (M00 + CCG), M56 (M00 + CGC), P16 (P00 + CC) and P17 (P00 + CG).

A set of simple PCR markers was developed and used to genotype the L × V or Su × V population. This set consists of 6 sequence characterised amplified region (SCAR) markers, 26 cleaved amplified polymorphic sequence (CAPS) markers and 1 derived CAPS (dCAPS: Neff et al. 1998). Primers for 16 of those SCAR and CAPS markers were developed based on DNA sequences of barley genomic clones publicly available in the GrainGenes databank; i.e. ABG-, BCD-, MWG-, Hor2 and Prx2. Primers for the 17 other SCAR and CAPS markers were developed based on DNA sequences of barley ESTs downloaded from the TIGR Gene Indices database (http://www.tigr.org/tdb/tgi/).

Those EST-based markers were named WBE- for Wageningen Barley ESTs. The primer design and polymorphism detection was done as described in Marcel and Niks (2004) using the Lasergene software package (DNASTAR Inc., Madison, WI, USA). Detailed information on the 33 SCAR and CAPS markers presented in this study is available in S1.

We used SSR markers to integrate the maps of the six barley populations. The L × V and the St × M populations were genotyped with 89 and 21 polymorphic SSR markers, respectively. The segregation data for 20 additional SSRs genotyped in St × M and 11 SSRs genotyped in OWB were obtained from Varshney et al. (2006a). The HV-, Bmac-, Bmag-, EBmac- and EBmag- markers were amplified according to the PCR protocols reported by Ramsay et al. (2000) and the GBMS- and GBM- markers according to the protocol described by Thiel et al. (2003). The primers were synthesised and the reverse primers IRDye-labelled at Biolegio BV (Nijmegen, The Netherlands). The PCR product was visualised on LICOR 4200 DNA sequencer. Additionally, a polymorphism test with 313 unmapped SSR primer combinations (GBM-) developed at IPK (Gatersleben, Germany) revealed 74 polymorphic markers in L × V and/or in Su × V (i.e. 24%). From these 74 SSRs, 13 pairs of markers were associated with the same consensus sequence resulting in a set of 61 unigene-based markers. Subsequently, we mapped 49 of those GBM markers in L × V and 12 in Su × V. The primer combinations of those 61 SSRs were unlabelled. Therefore, their PCR amplification products were fluorescently labelled according to the A/T labelling procedure before loading on gel.

A/T labelling procedure

DNA polymerases without proofreading activity generally catalyse the addition of a 3′-terminal deoxyadenosine

to a PCR amplification product (Clark 1988). This 3′ overhang of an adenosine residue in a PCR amplification product is widely used for universal cloning into a vector with a 3′-thymidine overhang (Magnuson et al. 1996; Zhou et al. 1995; Promega). Here, we used this strategy to fluorescently label PCR amplification products produced by SSR primer combinations with an adapter containing the appropriate IRDye to allow infrared detection during electrophoresis. The PCR amplification product of an SSR primer combination (5 µl) was ligated O/N at 37°C to the IRDye-labelled T-adapter in a ligation mixture containing 1 Unit *T4 Ligase* (Invitrogen), 1 pmol IRDye-700 labelled T-adapter, 2 nmol ATP, 0.25 Units *Supertaq* and 1.5 µl 5× T4-ligation buffer (Invitrogen) in a total volume of 10 µl. The T-adapter is generated by mixing equal amounts of the oligo's adT-top [$_{700}$GACTGCGTACC AATTCACT, near-infrared fluorescently labelled, (Biolegio, The Netherlands)] and adT-bot ($^P$GTGAA TTGGTACGCAGT$_{NH2}$). The bottom strand (adT-bot) contains a 5′-terminal phosphate group for efficient ligation and a 3′-terminal amine group to avoid A-tailing of the adapter.

### Construction of the barley consensus map

The quality of the data sets was estimated by running a Chi-square test for the segregation data of each marker. Then, we ordered twice markers within individual data sets with the program RECORD (Isidore et al. 2003). After each marker ordering by RECORD, conflicting data points (i.e. singletons) and other potential errors in the marker segregation data were identified and replaced by missing values as suggested by Isidore et al. (2003). A new improved version of Join-Map (JoinMap 4) based on a faster algorithm (Jansen et al. 2001), kindly provided by Dr. Van Ooijen (www.kyazma.nl), was subsequently used to calculate the six individual barley maps. Then, the integrative function of the software package JoinMap® 3.0 (Van Ooijen et al. 2001) was used to construct a framework map containing only the bridge markers identified between two or more populations. The map distances were calculated using the Kosambi mapping function. Next, the six individual barley maps were recalculated by adding the order of the framework markers, as given by JoinMap® 3.0, as a "fixed order file" into Join-Map 4. The final consensus map was calculated by using the framework map as fixed backbone onto which the unique loci of each individual map were added following the "neighbors" map approach described by Cone et al. (2002). The obtained consensus map was divided into 210 BINs of about 5 cM each.

For the sake of continuity of the system, we maintained as much as possible the BIN-defining markers of Kleinhofs and Graner (2001) in their role in the present map and they kept the same BIN number (e.g. 1H_01 for chromosome 1H BIN number 01). The BINs in the latter map span about 10 cM. Each 10 cM BIN was then subdivided into two 5 cM BINs in order to obtain a greater precision allowed by the high marker density of our map (e.g. 1H_01.1 and 1H_01.2).

### Nomenclature of the markers

The AFLP marker loci were assigned with a primer combination code followed by the fragment size as described by Qi and Lindhout (1997) and Bai et al. (2003). The nomenclature of the SSR markers was described in detail in Varshney et al. (2006b). The GBR-, GBS- and GBM- markers had been developed at IPK (Gatersleben, Germany) and correspond to Gatersleben Barley RFLP, SNP and microsatellite markers, respectively. The prefixes, "i", "m" and "d" were added to marker names to indicate isozyme markers, morphological markers and major disease resistance genes following a Mendelian segregation, respectively. Multiple segregating bands identified with a single probe or one primer combination were indicated with higher case letters for RFLP markers (e.g. ABC151A and ABC151B) and with lower case letters for SSR markers (e.g. Bmac0040a and Bmac0040b). The rest of the marker names remained unchanged compared to their record in GrainGenes 2.0.

### Mapping strategies of defence gene homologues (DGHs)

In the present paper we use the term "resistance gene" for genes that specifically confer a vertical resistance in race-cultivar-specific interactions, like *Rph-* genes to *P. hordei*. A "defence gene" is more generally induced in a plant response to a pathogen challenge, such as pathogenesis-related (PR) proteins. Analogues of resistance (RGA) and defence genes (DGA) are genes isolated using a PCR approach with degenerate primers designed from conserved domains of plant resistance and defence genes, respectively (Lanaud et al. 2004). Homologues of resistance genes (RGH) are genes identified by blast analysis that shares significant identity of the amino-acid sequence with known resistance genes (Monosi et al. 2004). Following the same nomenclature, we named the genetic markers derived from ESTs homologous to known defence genes Defence Gene Homologues (DGH).

A list of genes involved in the partial and/or basal resistance of plants to fungal pathogens was drawn up based on the information available in the scientific literature. We also considered the defence genes as well as a few resistance genes which were differentially expressed between susceptible and partial or non-host-resistant barley lines (Neu et al. 2003; Gjetting et al. 2004; Zierold et al. 2005; Jafary et al. unpublished data). We selected a total of 81 defence genes and five resistance genes that might explain the QTLs for partial resistance to barley leaf rust reported in this study (S2). The selected defence and resistance genes were tBlastx in the TIGR Gene Indices database. A barley EST was considered homologous to the gene used for tBlastx when annotated with a similar function in TIGR database and at a treshold *E* value $\leq 10^{-5}$. The blast analysis resulted in the identification of 245 homologous barley unigenes. For convenience, all the homologues of the 81 defence and five resistance genes selected are considered as DGH in this paper. Three strategies were followed to map a maximum of DGHs on the developed barley consensus map. The first strategy consisted in developing simple PCR markers based on the unigene sequences obtained by blast analysis in TIGR. In the second strategy, we searched the transcript map of barley, which is being developed at IPK, for mapped DGH sequences (Varshney et al. 2004). The third approach consisted simply in searching the literature for DGHs already placed on one of the maps used to construct the present consensus map.

### Disease evaluations at seedling plant stage

The long-time standard barley leaf rust isolate 1.2.1 (*P. hordei* Otth) was used to evaluate the level of partial resistance of the 150 DH lines of St × M and of the 94 DH lines of the OWBs at seedling stage in a greenhouse compartment. For St × M, the disease experiments were conducted in six replications in time and within each replication one seedling of each DH line was inoculated. For the OWBs, the disease experiments were conducted in three replications in time and within each replication three seedlings of each DH line were inoculated. The seeds were sown in trays of 37 × 39 cm, each of them containing two rows of 10–15 seeds. In each tray one seed of each parental line, Steptoe and Morex or *Dom* and *Rec* and of the control lines, L94 and Vada, were sown. The inoculation was performed as described by Qi et al. (1998b) with about 200 spores per $cm^2$. The latency period (LP) on each seedling was evaluated and the relative latency period (RLP50S) was calculated, relative to the LP on L94 (Parlevliet 1975).

### Statistical analysis

The pedigree analysis of Steptoe and of Morex was realised with the Peditree software package (Van Berloo and Hutten 2005). The wide sense heritability ($h^2$) for RLP50S was estimated from ANOVA in the St × M and the OWB populations according to the formula $h^2 = \sigma_g^2 / [(\sigma_g^2 + \sigma_e^2 / n)]$ where $n$ represents the number of replicates per line. ANOVA on RLP50S revealed significant genotype and replication effects in both populations. Therefore, the genotype effect of each line was extracted from the analysis of variance and its distribution tested for normality. The genotype effect was used to map QTLs on the skeletal maps "St × M basemap" and "OWBbase" (approximately 5–10 cM per marker interval) downloaded from the GrainGenes 2.0 website and from the OSU Barley Project website, respectively. The ANOVA was performed with the GenStat® 8.1 software package (VSN International Ltd. 2005). QTL-mapping was performed using MapQTL® 5.0 (Van Ooijen 2004) according to Qi et al. (1998b). A LOD threshold value of 3.1 was set for declaring a QTL (Van Ooijen 1999) and a two-LOD support interval was taken as a confidence interval for a putative QTL (Van Ooijen 1992). The Restricted-MQM program was run to estimate the proportion of explained phenotypic variance and the effect of the alleles from each parent.

The distribution of QTLs and DGHs on the consensus map was analysed by considering a BIN as "occupied" by a QTL when containing the corresponding peak marker or "occupied" by a DGH(s) when containing the corresponding molecular marker(s). A Chi-square test was realised to test the null hypothesis assuming independent distribution of BINs occupied with a QTL and BINs occupied with a DGH(s).

## Results

### A high-density consensus map of barley

We used barley SSR markers to link barley populations genotyped with RFLP and barley populations genotyped with AFLP markers. A barley consensus map was constructed, which integrates 3,258 markers. This new consensus map of barley covers a total genetic distance of 1,081 cM with an average distance between two adjacent loci of 0.33 cM. This is the highest density of markers reported for a barley genetic map to date. After the primary inspection of the data, 49 markers were removed because of their skewed segregation. From all singletons detected, 72% were

removed after the first marker ordering and the others 28% after the second marker ordering. The data set containing most singletons was the one of OWB with 2.3% of its total number of data points replaced by unknown values, while the data sets of Su × V, Su × CC, L × V, St × M and I × F contained respectively 1.4, 1.2, 0.5, 0.4 and 0.2% of singletons. One gap remained on chromosome 6H of the St × M map only. On the original St × M map (Hordeum-NABGMP1, GrainGenes 2.0) 42.7 cM separate the RFLP markers ABC170A and MWG798A at the telomeric end of 6HL. We tried to map markers within this interval to improve the map integration, but we did not succeed to reduce this gap to less than 30 cM. The framework map contained 50, 95, 89, 53, 70, 66 and 79 integrated bridge markers for the barley chromosomes 1H, 2H, 3H, 4H, 5H, 6H and 7H, respectively. It covered 1,028 cM with an average marker distance between two adjacent loci of 2.08 cM. The correctness of the final consensus map was evaluated by comparing the BIN markers order with the order of the same markers on the BIN map of Kleinhofs and Graner (2001). Marker orders between the maps were in good agreement with solely two inversions of markers on chromosome 3HS and at the distal end of chromosome 5HL. Chromosomes 3HS and 5HL were recalculated by adding the BIN markers of Kleinhofs and Graner (2001) as fixed order in Join-Map® 3.0. The full version of the consensus map is available as an Excel file in S3. The chromosomal assignment, the genetic position, the type of marker,

the BIN number and the map(s) of origin are given for each marker.

## Skewed segregation of molecular markers in six barley populations

Clusters of markers with skewed segregation were identified in all six barley linkage maps used for this study and on all seven barley chromosomes (Fig. 1). The distribution pattern of chromosomal regions associated with skewed marker segregation was different from one map to another. Xu et al. (1997) proposed to regard a chromosomal region as being associated with skewed segregation when four or more closely linked markers are significantly and consistently deviating from the 1:1 ratio. By following this proposition we associated approximately 75% of the consensus map with regions skewed in one or more population(s). The number of skewed markers varied from 10% of the markers mapped in the OWB population to 41% of the markers mapped in the I × F population. The I × F population also stood out by having the most extreme marker skewness, on chromosome 3H towards the alleles of Igri (allele B) (Fig. 1). In both Su × V and Su × CC map regions of skewed segregation were observed on all the seven barley chromosomes. It is remarkable that the markers were predominantly skewed towards the Vada allele (allele B) in Su × V while they were predominantly skewed towards the SusPtrit allele (allele A) in Su × CC.
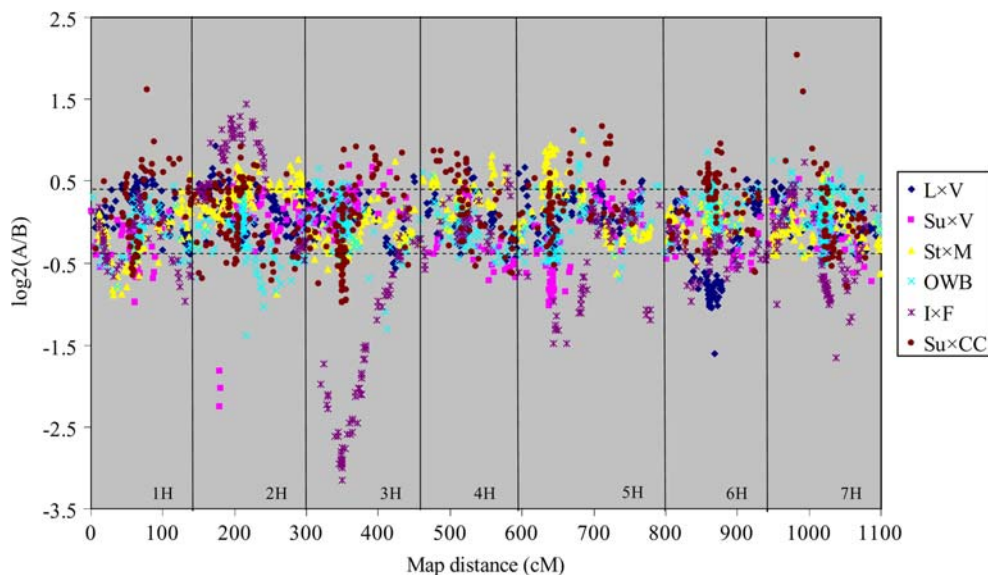


**Fig. 1** Scatter plot representing the distribution of marker skewness on the six individual barley maps, each *dot* representing one molecular marker. The chromosomes are represented by *solid vertical lines* along the map distance (*x*-axis) and their number is indicated. The log2 (*A/B*) (*y*-axis) is the log2 value of the ratio of the number of RILs carrying the allele of parental line *A* on the number of RILs carrying the allele of parental line *B*. Markers outside the two *dashed horizontal lines* are significantly skewed as calculated by Chi-square test

## Map position and characteristics of gene-targeted markers

A higher level of polymorphism was obtained with genomic SSRs than with genic SSRs. In L × V, 82% of the genomic SSRs tested (HVM-, Bmac-, Bmag-, EBmac-, EBmag- and GBMS-) and 52% of the genic SSRs tested (Hv*GeneName* and GBM-) were polymorphic while in St × M 81% of the genomic SSRs and 37% of the genic SSRs tested were polymorphic. A set of 61 GBM- SSR markers was mapped for the first time in this study. Those 61 markers were distributed over the 7 chromosomes which contained each between 5 and 14 of them (S4). Since the GBM primers have been developed on barley EST sequences, this new set of SSR markers represent 61 unique genes for which a map position is now available. The PCR-mixtures of the SSR markers analysed were successfully fluorescently labelled, following the A/T labelling procedure.

A list of 81 defence genes and 5 resistance genes that possibly explain the mapped QTLs for partial resistance was drawn up (S2) and tBlastx was executed in the TIGR Gene Indices database. For 33 of those genes, 63 barley homologues (S5) were mapped in one or more of the barley population(s) used to construct the consensus map. The number of those DGHs per chromosome ranged from 3 for chromosomes 1H and 4H to 18 for chromosome 2H. This suggests a very uneven distribution of the DGHs-based markers over the barley chromosomes. On average, less than one DGH per 45 cM was found on chromosomes 1H, 4H and 5H while one DGH per 8–15 cM was found on the four other chromosomes. Many of the mapped barley DGHs were organised in clusters composed of homologous genes. Those clusters occur for peroxidase like-genes on both arms of chromosome 2H (p*WIR3*, *Per2*, *Prx8* and *Prx2*), for beta-glucanase like-genes on chromosome 3H (*HvNR-R1* and *Glb33*) and for thaumatin like-genes on chromosome 7H (p*WIR232*).

## QTLs for partial resistance in seedlings of St × M and OWB

The wide sense heritability ($h^2$) for RLP50S was 0.83 in St × M and 0.84 in the OWBs. On the two populations the RLP50S values covered about the range between the susceptible line L94 and the partially resistant line Vada (Fig. 2). However, in both populations, the RLP50S for the parental lines were intermediate and similar to each other, indicating transgressive segregation, which implies that both parents contributed alleles for resistance. The genotypic effect used for QTL analysis followed a normal distribution in
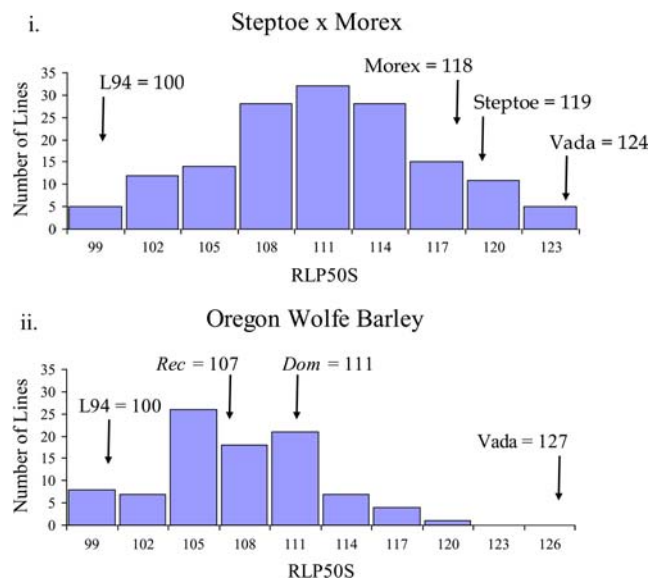


**Fig. 2** Frequency distribution of phenotypes for the relative latent period of leaf rust isolate 1.2.1 on seedlings (RLP50S) of the Steptoe × Morex population (**a**) and of the Oregon Wolfe Barley population (**b**). Values of the parental and control lines are shown by an *arrow*. The values indicated on the *x*-axis are the midvalues of each class

both populations, as expected in case of polygenic and quantitative resistance.

Four QTLs were detected in the St × M population and five in the OWB population (Table 2). Three of the nine detected QTLs were at a mapping position similar to a QTL reported by Qi et al. (1999, 2000) in two other mapping populations. We assume that they are at the same loci and provisionally use the gene designation of Qi et al: *Rphq8* in L × V (Qi et al. 1999) and *Rphq11* and *Rphq12* in L94 × 116-5 (Qi et al. 2000). The six other QTLs were at locations in which no QTL for resistance to *P. hordei* had been reported before. We designated them provisionally as *Rphq14* to *Rphq19*. In St × M, *Rphq11* on chromosome 2H and *Rphq14* on chromosome 1H had the greatest effect on the resistance, while in OWB *Rphq16* on chromosome 5H was the most effective QTL. The other QTLs contributed moderately to the level of partial resistance. Together, the QTLs identified explained 56 and 63% of the phenotypic variation in St × M and OWB, respectively. As expected from the transgressive segregation observed in Fig. 2, in both populations the two parents contributed QTLs with resistance alleles and QTLs with susceptible alleles (Table 2).

## Map-based selection of candidate genes to explain the QTLs

In this paper BINs were used to compare the position of 19 QTLs for partial resistance to barley leaf rust

**Table 2** Summary of QTLs conferring partial resistance against leaf rust isolate 1.2.1 at seedling development stage in two barley populations

| Steptoe × Morex | | | | | | Oregon Wolfe Barley | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| QTL | Chr. | cM[a] | LOD | Exp%[b] | Add[c] | QTL | Chr. | cM | LOD | Exp% | Add[d] |
| *Rphq8* | 7H | 86.5 | 3.4 | 4.1 | −1.16 | *Rphq12* | 2H | 124.4 | 5.7 | 5.6 | −1.11 |
| *Rphq11* | 2H | 95.1 | 21.0 | 34.1 | 3.31 | *Rphq16* | 5H | 160.0 | 13.9 | 32.7 | 2.70 |
| *Rphq14* | 1H | 11.6 | 9.6 | 12.9 | −2.00 | *Rphq17* | 3H | 52.2 | 6.6 | 10.6 | 1.55 |
| *Rphq15* | 6H | 25.1 | 5.3 | 5.5 | 1.31 | *Rphq18* | 2H | 53.6 | 5.1 | 6.9 | −1.22 |
| | | | | | | *Rphq19* | 4H | 57.5 | 4.2 | 7.6 | −1.32 |
| Total | | | | 56.6 | 1.46 | Total | | | | 63.4 | 0.60 |

[a] Position of the peak marker on the consensus map (in centiMorgan)

[b] Proportion of the explained phenotypic variance

[c] Additive effect of the allele from Steptoe; an effect of 1 is equivalent to a prolongation of the latency period of the rust fungus of 1.72 h; a negative sign indicates that the resistance allele has been contributed by Morex

[d] Additive effect of the allele from *Dom*; an effect of 1 is equivalent to a prolongation of the latency period of the rust fungus of 1.44 h; a negative sign indicates that the resistance allele has been contributed by *Rec*

with the position of 63 DGHs possibly involved in the defence of plants to fungal pathogens (Fig. 3). Nine of the QTLs were detected in this study on the St × M and OWB populations while the other ten QTLs had been detected previously on L × V (Qi et al. 1998b, 1999), Su × V (Jafary et al. 2006a) and Su × CC (Jafary et al. 2006b; including 116-5 × L94, Qi et al. 2000). An identical name was assigned to QTLs mapped in two or more populations which had overlapping confidence intervals. A BIN containing the peak marker of a QTL was considered as "occupied". Since a QTL mapped in several populations usually had in each population a different peak marker, one QTL could occupy more than one BIN. Similarly, a BIN containing one or more DGH(s) was considered as "occupied". The 19 QTLs occupied 21 BINs and the 63 DGHs occupied 43 BINs. Eight BINs were co-occupied by a QTL and by a DGH(s): *Rphq6* with WBE105 (peroxidase); *Rphq18* with Pox, GBR1062, GBR0126 (peroxidase), GBR0239 (Lipid transfer protein) and GBS0864 (WIR1 protein homologue); *Rphq2* with Prx2, WBE111 and GBR1182 (stress-related peroxidase); *Rphq3* with WBE103, GBS0164 (superoxide dismutase) and with WBE201 (serine/threonine-protein kinase Pelle); *Rphq1* with GBR0202 (PR-1 protein); *Rphq8* with WBE101 (HvNR-F1); *Rphq9* with GBR0192 (LR10 resistance like-protein). *Rphq4* and WBE108 (thaumatin like-protein) were mapped next to each other in the consecutive BINs 5H_02.1 and 5H_02.2, which was considered as a case of co-occupation.

We tested by Chi-square test the null hypothesis assuming an independent distribution of BINs occupied by a QTL and BINs occupied by a DGH(s) (Table 3). The null hypothesis was not rejected,

suggesting an independent distribution of the QTLs and DGHs over the consensus map of barley. Nevertheless, we observed more (8 vs. 4.3) BINs in which a peak marker coincided with a DGH than expected, but the association was not strong enough to reject the null hypothesis of independent distribution.

## Discussion

### Properties and usefulness of the high-density consensus map of barley

The final consensus map comprising all 3,258 markers was calculated by combining the use of traditional software packages (Excel, JoinMap® 3.0) with the use of recently developed software packages (RECORD, JoinMap 4). The marker order was always under control of fixed-order files, extracted from the framework map, to guarantee that the integrated marker order remained in agreement with the marker order as observed in the individual maps. The alignment of the individual maps calculated in JoinMap 4 without fixed-order files revealed very few and limited marker reor-

**Fig. 3** Locations of 19 QTLs for partial resistance to barley leaf rust mapped in five individual barley linkage maps and of 63 defence gene homologues (DGH) on the BIN map extracted from the constructed high-density consensus map of barley. Length of QTL bars corresponds approximately to the two LOD support intervals (from peak marker) based on the results of MQM. The loci preceded by an *asterisk* are BIN markers, the 61 loci *underlined* are new genic SSRs and the 63 loci in **bold** are DGH-based markers. Numbers on the left side show the distance in centiMorgans (according to Kosambi) from the top of each chromosome. The full consensus map is available as an Excel file in S3

dering between the maps (data not shown). This is an indication that the marker order is very stable, which can only be achieved when the data are almost free of errors. We believe that the visual inspection of the data sets for the identification of errors and replacement of singletons by missing values, as proposed by Isidore
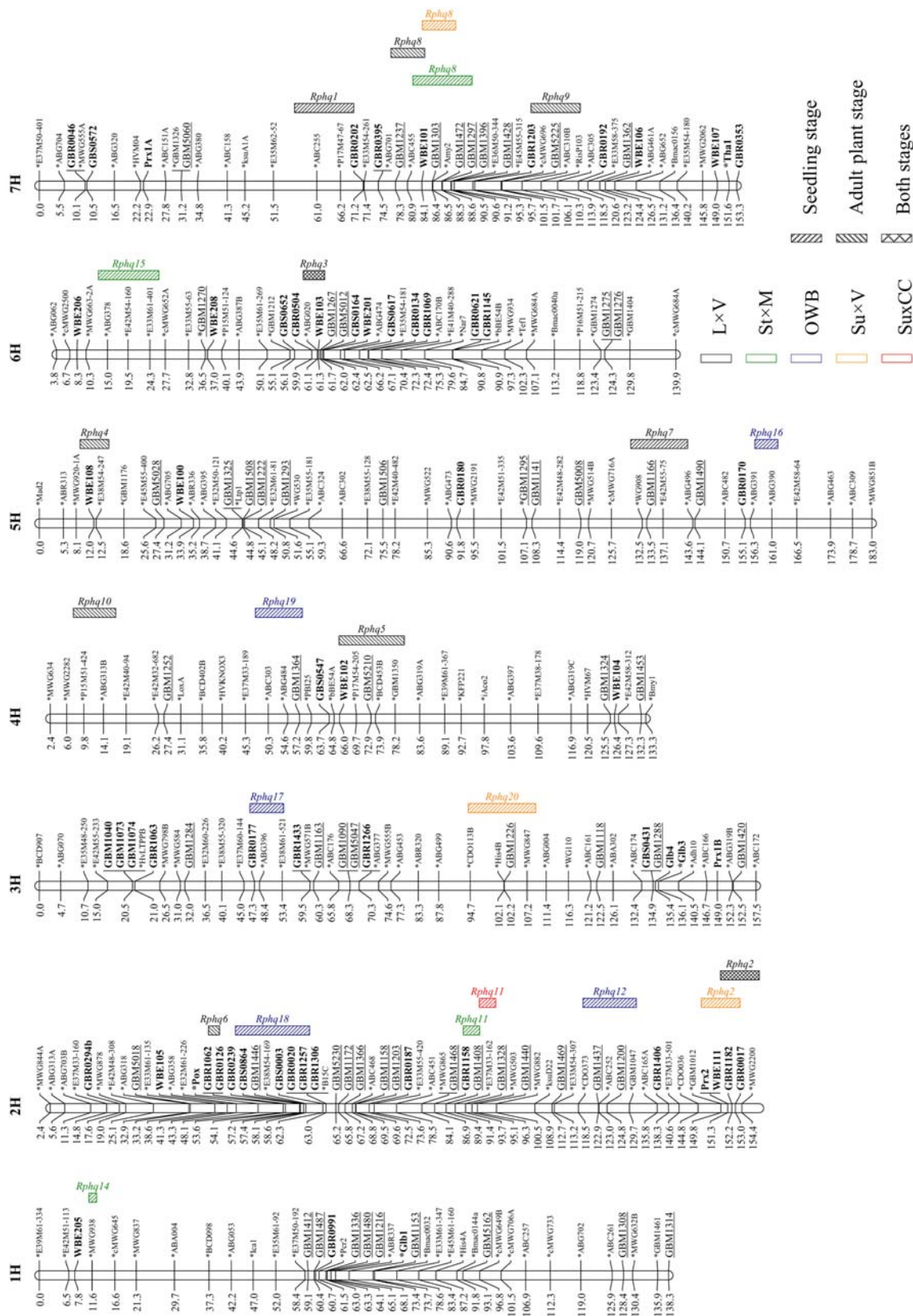
et al. (2003), plays a significant role in the stability of the marker order.

AFLP and RFLP markers are the most abundant marker types on this high-density consensus map, respectively 60 and 26% of the total number of markers. The SSR marker system was used to map bridge markers between the populations mainly genotyped with AFLP markers (L × V, Su × V, Su × CC and OWB) and the ones mainly genotyped with RFLP markers (St × M and I × F). SSR markers represent 11% of the total number of markers on the consensus map. Both RFLP and SSR markers are highly transferable between populations of the same species but also between species of the same family. On the other hand, the transferability of AFLP markers is limited to the same plant species. However, even if common AFLP markers can be identified among populations (Qi and Lindhout 1997) and used to align genetic maps (Rouppe van der Voort et al. 1997), the transferability of AFLP markers among laboratories remains disputable. We took as criteria for the selection of potentially common AFLP markers across populations the co-migration of amplification products obtained with identical primer combinations and the localisation of markers to similar map positions. Between the L × V, Su × V and Su × CC linkage maps, developed in the same laboratory (Laboratory of Plant Breeding, Wageningen University), 271 AFLP markers were polymorphic in at least two of these populations. Only 3 out of 1,362 AFLP markers mapped in the three populations at Wageningen University were unambiguously in common with the 594 AFLP markers mapped in the OWB population at the Oregon State University. The barley populations developed at Wageningen shared common parental lines (Vada or SusPtrit) and were genotyped with at least 17 identical primer combinations while the OWB population had no parental line in common with the other populations and was genotyped with only 8 primer combinations identical to one of the three other AFLP maps. This can only partly explain the near absence of common AFLP markers identified between the maps developed in different laboratories. We assume that differences in the assessment of fragment sizes of AFLP bands by different laboratories are mostly responsible for the lack of common markers identified. Differences in assessed sizes could result from the use of a different visualisation system, size ladder or scoring methodology. The generation of reference AFLP fingerprints including parental lines from the populations involved and making them publicly accessible can further enhance the identification of common markers between unrelated barley mapping populations studied at different laboratories.

**Table 3** Chi-square test on the probability of independent distribution of QTLs and DGHs over BINs on the consensus genetic map of barley

| Class[a] | | Observed results ($O$) | Expected results ($E$) | $(O-E)^2/E$ |
|---|---|---|---|---|
| QTL | DGH | | | |
| 0 | 0 | 154 | $(189 \times 167)/210 = 150.3$ | 0.091 |
| 0 | 1 | 35 | $(189 \times 43)/210 = 38.7$ | 0.353 |
| 1 | 0 | 13 | $(21 \times 167)/210 = 16.7$ | 0.819 |
| 1 | 1 | 8 | $(21 \times 43)/210 = 4.3$ | 3.183 |
| | | | | $\chi^2 = 4.446$[b] |

[a] A class 0 indicates BINs unoccupied by QTL and/or DGH and a class 1 indicates BINs occupied by a QTL and/or by one to several DGH(s)

[b] With a number of degrees of freedom ($df$) = 3 the null hypothesis is accepted with a probability $0.1 < P < 0.5$

The lack of polymorphism observed on chromosome 6H of the St × M map over 30 cM may be due to sharing a common ancestor by the two parents. The pedigree analysis of Steptoe and of Morex revealed that they share five barley lines in their ancestry: Eckendorfer, Frew. Berg, Schladener I, Schwarze and Titan. We presume that the lack of polymorphism on 6HL is indeed due to shared ancestry.

Approximately 75% of the consensus map was associated with regions of skewed segregation in one or more of the six integrated populations. In this study, no difference was observed between the skewness of marker segregation from the DH and from the RIL populations; i.e. respectively 24 and 22% of markers showing skewed segregation. This does not support the observation of Xu et al. (1997) who reported significantly higher frequencies of skewed markers in RIL populations than in other population structures. Skewed segregation may arise from genetic, physiological and/or environmental causes and the relative contribution of each of these factors may depend on parental combination and factors during the development of the mapping population (reviewed in Xu et al. 1997).

Optimising the mapping of gene-targeted markers in plants

The sequence data generated by large-scale EST projects has made it feasible to develop molecular markers directly from genes rather than from anonymous DNA fragments. The development of gene-targeted markers (GTM) (Andersen and Lübberstedt 2003) is particularly relevant in plant species like barley for which genome sequencing cannot be completed at short term and for which a large number of ESTs is available. The ongoing development of genetic maps based on GTMs,

also called transcript maps, in barley by Varshney et al. (2004), Sato et al. (2004) and Rostoks et al. (2005) has already produced sets of 1,051 (RFLP, SNP, SSR), 1,055 (SNP) and 323 (SNP, SSR) GTMs, respectively. In this study, we contributed 75 new GTMs to the barley community, 61 SSRs (GBM-markers) and 14 CAPS and SCAR markers (WBE-markers), which will serve to improve the available barley transcript maps. The conversion of expressed sequence information into molecular markers with a position on a linkage map is a laborious and costly process. In order to minimise the effort and to avoid the mapping of redundant ESTs from one laboratory to another it would be advisable to integrate all contributions on one public transcript map. The construction of such a high-density consensus barley linkage map, integrating the individual linkage maps used to map GTMs, could be achieved with the methodology that we applied in this paper.

Among the different marker technologies available to develop GTMs, genic SSRs have proven as markers of choice for their high quality and the robustness of their amplification patterns along with their multiallelic nature, codominant inheritance and superiority in terms of transferability and comparative mapping in related species (Varshney et al. 2005; Parida et al. 2006). Nevertheless, SSR markers often produce a complex mixture of PCR products that requires high-resolution separation on polyacrylamide gels. The direct synthesis of a fluorescently labelled primer is about five times more expensive than the synthesis of an unlabelled primer. The use of tailed primer labelling to label PCR product also results in extra costs due to the elongated size of the tailed primers and to the requirement of a second PCR. The economic aspect becomes especially relevant when a large number of primer combinations has to be tested on a small number of individuals. In this study, 313 unlabelled primer combinations were screened between the parents of two mapping populations and 61 new genic SSR markers were mapped. We optimised this extensive SSR analysis by fluorescent labelling of unlabelled PCR-mixtures followed by size-separation on polyacrylamide gels. A procedure referred to as A/T labelling. Subsequently, the primer combinations amplifying markers of interest for high throughput applications can be directly synthesised with a fluorescent label. Automated sequencers are widely used for DNA sequencing, SSR analysis, AFLP analysis and reverse genetics. These sequencers are perfectly suited for the high-resolution size separation, detection and analysis of PCR products. We presume that the A/T labelling procedure can also be applied with other fluorescent dyes.

## Distribution of QTLs for partial resistance to leaf rust on the barley genome

The level of partial resistance to leaf rust among spring barley germplasm is not only high but also increasing due to selection against high levels of susceptibility by the breeders (Niks et al. 2000). The continued increase of levels of partial resistance in modern barley germplasm implies that there is an abundance of loci carrying such genes. The present study supports this assumption. Each parental barley combination segregates for different sets of QTLs, with only few QTLs shared by any pair of cultivars. In total, 19 QTLs were placed on the present barley consensus map. Those results confirm the earlier observations of Qi et al. (2000) and show that the abundance of QTLs for partial resistance is a reality.

## Significance of the candidate gene analysis for cloning a QTL

The main challenge of GTMs development is to associate sequence polymorphisms with phenotypic variation. Several authors already mapped QTLs on linkage maps that contain GTMs (Chen et al. 2001; Faville et al. 2004; Pajerowska et al. 2005). This may allow the identification of associations between markers that are based on genes with known or putative function and QTLs for agronomic traits. The candidate gene approach has often been used to characterise disease resistance loci. Numerous genes involved in pathogen recognition, signal transduction and defence have been isolated. Traditionally, analogues of those resistance (RGA) or defence genes (DGA) are used to identify candidate genes (Pflieger et al. 2001; Lanaud et al. 2004). More recently, a procedure based on the selection of homologues of genes involved in plant defence by blast analysis was applied to identify candidate genes (Pajerowska et al. 2005). We propose to name those genes Defence Gene Homologues (DGH). In this study, eight BINs were co-occupied by a QTL and by a DGH(s) involving genes that encoded receptor-like kinase (RLK), *WIR1* homologues and several defence response genes like peroxidases, superoxide dismutase and thaumatin. Those results indicate striking similarities with previous reports, where genes with such functions also tended to co-localise with QTLs for disease resistance in wheat and in rice (Faris et al. 1999; Wang et al. 2001; Ramalingam et al. 2003). In wheat, the *WIR1* gene has a function in increasing the adhesion of the membrane to the cell wall in case of pathogen attack (Bull et al. 1992). In barley, *WIR1* and *WIR1* homologues were found to be induced upon

inoculation with the host pathogen *Blumeria graminis* f. sp. *hordei* (Jansen et al. 2005; Zierold et al. 2005) and with the non-host pathogen *P. triticina* (Neu et al. 2003). It is often assumed that DR genes like those encoding peroxidase (PR-9), superoxide dismutase and thaumatin-like protein (PR-5) are potential candidates to explain the QTLs for quantitative resistance to plant pathogens. Peroxidase ($H_2O_2$) and superoxide dismutase ($O_2^-$) are reactive oxygen intermediates (ROIs). ROIs have been implicated in signal transduction as well as in the execution of defence reactions such as cell wall strengthening and a rapid hypersensitive reaction (reviewed in Hückelhoven and Kogel 2003). But the role of ROIs in the establishment and maintenance of either host cell inaccessibility or accessibility during attack by a fungal plant pathogen is not yet fully understood. The vacuolar peroxidase *Prx7* was implicated as a susceptibility factor in the response of barley to attack by *B. graminis* f. sp. *hordei*, enhancing successful haustorium formation (Kristensen et al. 2001). *Prx7* mapped in the same region of chromosome 2HL (Giese et al. 1993) as *Prx2* which is another peroxidase gene locus identified as a candidate to explain *Rphq2* in this study. The mildew haustorium promoting effect of *Prx7* (Kristensen et al. 2001) qualifies peroxidase genes as candidates for QTLs for partial resistance to *P. hordei*.

However, it always remains to be determined whether the candidate gene and the QTL map in the same position on the linkage map by chance or indeed because the candidate gene really is responsible for the phenotype determined by the QTL. For instance, many of the mapped barley DGHs were organised in clusters composed of homologous genes. DR gene families are often organised in complex loci as described by Muthukrishnan et al. (2001). So the fact that a DGH is co-segregating with a QTL does not mean that this DGH is the gene underlying the QTL. Remarkably, a cluster of DGHs mapped in the centromeric region of chromosome 6H was composed of homologues of genes from very different families like At4g22240, pBI-1, Sod, HvNR-F6 and PAL. This region of chromosome 6H might represent a gene rich region. We also performed a Chi-square test which showed that the distribution of the 19 QTLs for partial resistance to barley leaf rust was independent from the distribution of the 63 DGHs mapped on the present consensus map. This suggests that most of the presently investigated DGHs are not responsible for the phenotype determined by the QTLs and that co-segregation between a DGH and a QTL is likely to occur by chance. At the end, fine-mapping experiments are necessary to locate precisely the implicated candidate gene and the QTL locus. Transcrip-

tome profiling can confirm the involvement of the gene in the biochemical pathway leading to the phenotype observed but will not demonstrate conclusively whether the candidate gene is the gene determining the trait variation in the mapping population. The evidence that a candidate gene is really responsible for the trait variation can be definitively demonstrated by genetic transformation experiments.

Note

All the mapping data and segregation data of the three RIL populations, L94 × Vada, SusPtrit × Vada and SusPtrit × Cebada Capa, used to construct the high-density consensus map of barley, have been deposited in the GrainGenes 2.0 database.

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