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QTL mapping provides evidence for lack of association of the avoidance of leaf rust in Hordeum chilense with stomata density

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Abstract In cereals, rust fungi are among the most harmful pathogens. Breeders usually rely on short-lived hypersensitivity resistance. As an alternative, "avoidance" may be a more durable defence mechanism to protect plants to rust fungi. In *Hordeum chilense* avoidance is based on extensive wax covering of stomata, which interferes with the induction of appressorium formation by the rust fungi. High avoidance levels are associated with a higher stoma density on the abaxial leaf epidermis. The avoidance level was assessed as the percentage of germ tube/stoma encounters that did not result in appressorium differentiation by *Puccinia hordei*, the barley leaf rust fungus. One hundred F_2 individuals from the cross between two *H. chilense* accessions with contrasting levels of avoidance showed a continuous distribution for avoidance of the rust fungus and for stoma density, indicating quantitative inheritance of the traits. No significant correlation was found between avoidance and stoma density in the segregating F_2 population. In order to map quantitative trait loci (QTLs) for both traits, an improved molecular marker linkage map was constructed, based on the F_2 population. The resulting linkage map spanned 620 cM and featured a total of 437 AFLP markers, thirteen RFLPs, four SCARs, nine SSRs, one STS and two seed storage protein markers. It consisted of seven long and two shorter linkage groups, and was estimated to cover 81% of the *H. chilense* genome. Restricted multiple interval mapping identified two QTLs for avoidance and three QTLs for stoma density in the abaxial leaf surface. The QTLs for avoidance were mapped on chromosome 3 and 5; those for stoma density on chromosomes 1, 3 and 7. Only the two QTLs regions located on chromosome 3 (one for avoidance and the other for stoma density) overlapped. The wild barley *H. chilense* has a high crossability with other members of the *Triticeae* tribe. The knowledge on the location of the QTLs responsible for the avoidance trait is a prerequisite to transfer this favourable agronomic trait from *H. chilense* to cultivated cereal genomes.

Keywords Avoidance · Stoma density · Barley leaf rust · Genetic linkage map · QTL mapping

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Introduction

Fungi belonging to the group of rust fungi cause the most serious and widespread diseases in cereals (Buchenauer 1982). The cereal rusts occur worldwide and produce frequent severe epidemics with substantial annual yield losses. About 10% of the world grain crop per year is lost to rust infections (Agrios 1997).

Until recently, breeders concentrated on the use of single race-specific resistance genes conferring a hypersensitive reaction of plant tissue to cereal rusts. Such a use of resistance often results in ephemeral disease control, because virulent genotypes are rapidly evolving from the pathogen population (Mundt and Browning 1985). Rubiales and Niks (1992b, 1996) identified an alternative defence mechanism in *Hordeum chilense* Roemer et Schultes ($2n$=14), a perennial wild barley occurring in Chile and Argentina. This alternative defence is designated "avoidance". Avoidance reduces the chance of intimate contact between the plant tissue and the parasite. It is different from host and non-host resistance, which operates after the parasite has come into intimate contact with the plant cells (Parlevliet 1981). The avoidance in *H. chilense* is characterised by the rust germ tubes overgrowing the stomata rather than forming an appressorium and penetrating the stomata to invade the leaf tissue (Rubiales and Niks 1992b, 1996). The avoidance in *H.*

chilense is effective against several leaf rust species, such as *Puccinia hordei*, *P. triticina*, *P. recondita* and *P. agropyrina* (Rubiales and Niks 1992a, 1996). On plants with high avoidance, relatively few sporelings will produce an appressorium and penetrate into the mesophyll tissue. Only then will possible host or non-host resistance mechanisms, such as the failure of haustorium formation, become apparent. Since rust fungi often have no difficulty finding and penetrating stomata on non-host plant species that have a morphology similar to their host (Niks 1986), avoidance is by no means a character that occurs on all non-host plant-pathogen combinations.

This avoidance of rust fungi has only been found in *H. chilense* and in four other wild species of barley (*H. brachyantherum*, *H. marinum*, *H. parodii* and *H. secalinum*) (Rubiales et al. 1996).

In previous studies on collections of *H. chilense* accessions, a high positive correlation was found between avoidance level on the adaxial epidermis and stoma density on the abaxial epidermis of the leaf (Rubiales and Niks, 1996; Vaz Patto et al. 2001). High avoidance is associated with an extensive wax covering of the stomatal guard cells (Rubiales and Niks 1996), perhaps decreasing gas exchange through the stomata. Rubiales and Niks (1996) suggested that the high stoma density in lines with high avoidance might be a provision to compensate for the presumed less efficient gas exchange by wax-covered stomata. These authors suggested that the association between avoidance level and stoma density might either be due to pleiotropy, to genetic linkage or to linked evolution of both traits over ecotypes. The latter idea has been supported by more recent evidence (Vaz Patto et al. 2001).

H. chilense has been used by cereal breeders because of its agronomically interesting characteristics and its high crossability with *Triticum*, *Hordeum*, *Secale* and *Agropyron* (Martín and Chapman 1977; Martín et al. 1996, 1998, 1999). The amphiploid hybrids with cultivated barley (*Hordeum vulgare*) are unfortunately sterile, but those with various wheat species are fertile. The amphiploid *H. chilense* × *T. turgidum* conv. *durum*, called tritordeum, is being used in a breeding programme as a new crop or as bridge to transfer useful genes from *H. chilense* to wheat (Martín and Cubero 1981; Martín et al. 1998). Genes governing avoidance would be among the useful genes to be transferred to modern cereal cultivars.

Restriction fragment length polymorphisms (RFLP) have been used in combination with random amplified polymorphic DNA (RAPD), simple sequence repeats (microsatellites or SSR) and sequence-characterised amplified region (SCAR) markers to generate a preliminary *H. chilense* map (Hernández et al. 2001). RFLP probes have proven their effectiveness for alignment of the *H. chilense* chromosome $2H^{ch}$ map with a consensus map of the group 2 of wheat, suggesting that colinearity is maintained for this chromosome among these two species (Hernández et al. 2001). However, the requirement of a relatively large amount of DNA and the labour and time-consuming technology of Southern hybridisation that has to be repeated for each RFLP marker set strong limitations to its use in linkage mapping and marker-assisted selection. Fast and easy polymerase chain reaction (PCR)-based markers like microsatellites and RAPDs, which require lower amounts of DNA, are a more logical choice for marker-assisted selection. The requirement of a priori sequence information to design specific microsatellite primers and the poor reproducibility of RAPDs (Penner et al. 1993) made us to consider AFLP marker technology (Vos et al. 1995) in order to generate a dense marker linkage map of *H. chilense*.

The development of this dense molecular marker linkage map would allow the quantitative trait variation to be dissected into the effects of individual genome regions, the quantitative trait loci (QTLs), that are identified by linkage to markers on the marker map (e.g. Paterson et al. 1988). Determination of the number, location, and magnitude of effects of QTLs is important for improving breeding and selection efficiency.

In the investigation reported here, we improved the preliminary *H. chilense* map (Hernández et al. 2001) and extended it with 437 AFLP markers. We established the inheritance of the avoidance of rust fungi and of stoma density and the association between them and identified QTLs that contribute to these two traits.

Materials and methods

Plant material

Hordeum chilense accession H7, showing a high level of avoidance of rust fungi and high stoma density on the abaxial epidermis of the leaf, and accession H1, with a low level of avoidance and a low stoma density on the abaxial leaf epidermis (Rubiales and Niks 1996), are representatives of two distinct ecotypes of *H. chilense* (Vaz Patto et al. 2001). An F_2 population consisting of 100 plants obtained from the cross H1×H7 was used for molecular marker analysis to construct a linkage map that was integrated with the preliminary map of Hernández et al. (2001). The same population was tested for avoidance and for stoma density in the greenhouse with the two parental *H. chilense* accessions as controls.

Phenotypic evaluation

The F_2 plants were raised at the Instituto de Agricultura Sostenible, Córdoba, Spain, in small pots (1,000 cm^3) in a greenhouse compartment and maintained at 18–25 °C and ambient humidity. Per F_2 plant, one clonal offspring plant was transferred to the Wageningen University, The Netherlands, and kept under similar growing conditions.

From each plant, two tillers were chosen, the sixth leaves of which (counted from the base) were inoculated with *Puccinia hordei* isolate 1-2-1, which is a monospore culture derived from 1-2 (Parlevliet 1976), maintained at the Laboratory of Plant Breeding, Wageningen University. The leaves were fixed horizontally with their adaxial surface facing up, and 6 mg of *P. hordei* spores (resulting in approximately 400 spores/cm^2) was applied using a settling tower.

The sampling of the leaves and the staining with Uvitex for histological observations was as described by Rubiales and Niks (1996). Observations were made with an epifluorescence microscope (Nikon Fluophot, V-exitation, 380–425 nm) at 100× magnification.

The avoidance level was measured as the percentage of germ tube/stoma encounters that did not result in appressorium formation.

Stomatal densities were determined on the abaxial epidermis of leaf segments of the F_2 plants (three microscope fields per leaf, 2.6 mm^2 each, white light, 100× magnification) in the same two leaves per plant that were used to determine the level of avoidance.

Four replicate experiments were conducted in different times of the year, each with two leaves per F_2 plant.

Wax covering of the stoma

The leaves of the contrasting parental accessions (H1 and H7) and of ten F_2 plants, selected to represent the full range of avoidance levels were observed by scanning electron microscope (SEM) to determine the epicuticular wax covering of the stomata. Samples of the leaves were mounted, with the adaxial surface facing up, on metal stubs and frozen in liquid nitrogen. The specimens were sputter-coated with gold and examined and photographed in the scanning electron microscope (JEOL 35C at PCM Wageningen University) at an accelerating voltage of 15 kV.

Map construction

RFLP, SCAR, STS, microsatellite and seed storage protein markers

The preliminary map was based on the same H1×H7 F_2 population, and contained 16 RFLP markers, four SCARs, one STS (sequence tagged site, Xabc465) and 13 SSR microsatellite markers and two seed storage protein markers (Hernández et al. 2001). These data were available to be integrated with the AFLP marker data collected in the present work.

The AFLP protocol

DNA was extracted from leaf tissue of not fully expanded leaves according to the CTAB method (Van der Beek et al. 1992). The AFLP procedure was performed as described by Vos et al. (1995) with modifications by Qi and Lindhout (1997).

A total of 32 *Eco*RI/*Mse*I (+3/+3) primer combinations, chosen from Qi and Lindhout (1997), were applied to AFLP fingerprint the parental lines (H1 and H7) in order to select the most informative primer combinations. This resulted in the selection of 15 primer combinations – E32M55, E32M61, E32M62, E33M55, E35M48, E35M49, E35M54, E35M55, E38M54, E38M55, E38M59, E38M61, E42M47, E42M48, E42M50 – that were applied on the mapping population and on the set of addition and substitution lines. Primer core sequences were according to Vos et al. (1995). AFLP bands were detected by radioactive labelling.

Clearly readable amplified fragments were visually scored predominantly as dominant genetic markers, i.e. as presence or absence of the band. To identify possible AFLP band pairs that could be used as co-dominant markers, we followed the same genetic criteria as in Alonso-Blanco et al. (1998). Two AFLP bands were considered to be allelic when they are derived from two different parents, generated by the same primer combination and segregate as one locus; i.e. in all progeny one or both parental bands are present, but in none of the progeny are both bands absent. In addition to this, both bands in the putative heterozygous individuals should have a weaker intensity than the single band in the putative homozygous individuals.

To ensure accuracy, we scored all AFLP markers at least twice independently, and ambiguous marker bands were recorded as unknown in the data set.

The AFLP amplification products were designated according to Qi and Lindhout (1997).

Data analysis and map construction

Linkage analysis and segregation distortion tests ($P \leq 0.05$) were performed using JOINMAP version 2.0 (Stam 1993; Stam and Van Ooijen 1995). The determination of linkage groups of markers was done with a LOD score of 4.0, with the exception that one marker [HVM54(2HL)] was assigned to its position on chromosome 2 map at a LOD of 3.5. The calculations of the linkage maps were done using all pairwise recombination estimates lower than 0.45 and a LOD score higher than 0.1 and using the Kosambi mapping function (Kosambi 1944).

Linkage groups were assigned to the corresponding *H. chilense* chromosome using H1 addition and substitution lines of wheat. AFLP bands were considered specific for *H. chilense* chromosomes if they were present in one of the addition lines and in H1, but absent in *T. aestivum* parent Chinese Spring and absent in H7. In this way, linkage groups of linked AFLP markers were assigned to the specific *H. chilense* chromosome.

After running the program with the entire data set, six AFLP markers, one RFLP and one SSR marker were identified that showed association with two separate linkage groups at a rather high LOD score. They disturbed a correct grouping of markers into the expected seven linkage groups. Such markers represent probably different DNA fragments with identical electrophoretical mobility, leading to superimposed marker bands on the gels. These markers were removed from the data set.

QTL mapping

From the improved linkage map, a 'base map' was extracted and used for QTL identification. The base map was obtained from the original map with the exclusion of 86 redundant markers clustered at the same position. All of the codominant markers were kept as well as co-segregating dominant markers if they complemented each other for missing values in the mapping population. The base map contained 380 instead of 466 markers.

Analysis of variance was carried out for avoidance and stoma density on the F_2 population, and broad-sense heritability (h_b^2) was calculated. The similar LOD profiles obtained with the data from each of the four replications justified pooling the data from the four replications for QTL mapping.

A computer software package, MAPQTL version 4.0 (Van Ooijen and Maliepaard 1996), was used for interval mapping (Lander and Botstein, 1989) and restricted multiple QTL mapping (Jansen 1993; Jansen and Stam 1994). In the regions of the putative QTLs, the markers with the highest LOD values were taken as co-factors. When LOD values for some markers in other regions became significant, they were gradually added as co-factors, until the LOD profile stabilised. LOD values of 3.7 (avoidance) and 3.4 (stoma density) were chosen by the permutation test (available in the MAPQTL package) as significant threshold values for declaring a QTL at 95% confidence. The additive effect and the percentage of total phenotypic variation explained by each putative QTL were also estimated using MAPQTL software.

Results

Phenotypic data

The mean values of avoidance level of the two parental accessions were 23% (SD=6.5) and 94% (SD=3.8) germtube/stoma encounters not resulting in appressorium formation (Fig. 1a), and stoma density averaged 5.4 (SD=5.7) and 30.2 (SD=16.6) stoma/mm^2, respectively (Fig. 1b). The F_2 population showed a large and continuous variation in avoidance and stoma density. The Kolmogorov-Smirnov statistic test showed that

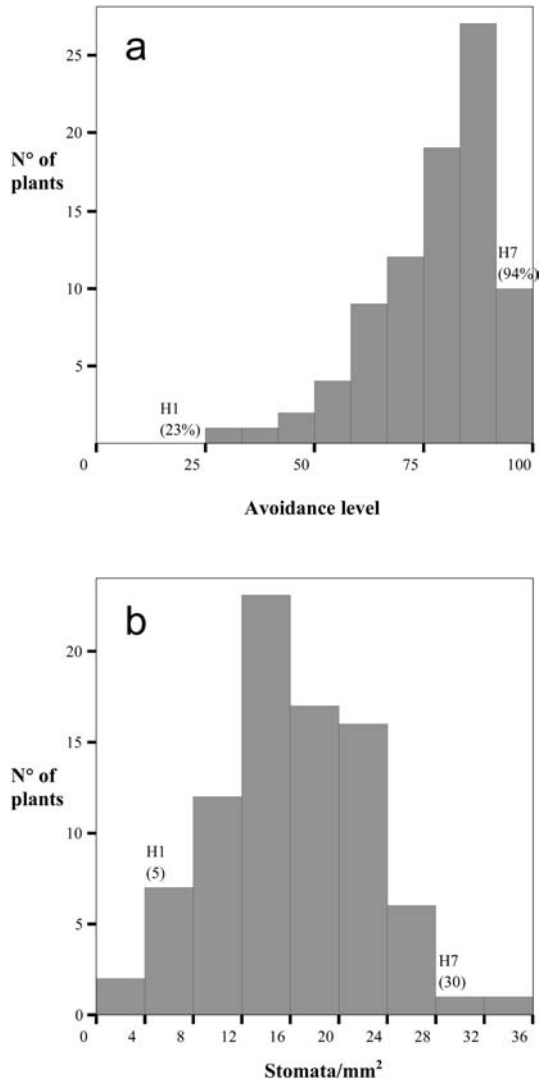


Fig. 1 Frequency distribution of (**a**) the avoidance level measured on the adaxial leaf epidermis as the percentage of the germ tube/stoma encounters that did not result in an appressorium and of (**b**) the stoma density on the abaxial leaf epidermis, (averages over four experiments) in the *Hordeum chilense* F_2 population H1×H7. The average levels of avoidance and stoma density of the two parental accessions (H1 and H7) are indicated

avoidance level had no normal distribution ($P<0.0001$), while stoma density was normally distributed ($P=0.2$). An arcsine square root transformation was applied to the avoidance level data in order to improve homogeneity of residual variance.

The phenotypic values of the two parental accessions were not significantly different from the values of the most extreme F_2 plants ($P \geq 0.2$), so no transgressive segregation was observed for either of the traits.

The broad-sense heritability for avoidance was 0.56 and for stoma density, 0.49. No significant correlation was found between the stoma density and the level of (untransformed) avoidance ($r = -0.28$, Fig. 2), indicating that the two traits inherit independently.

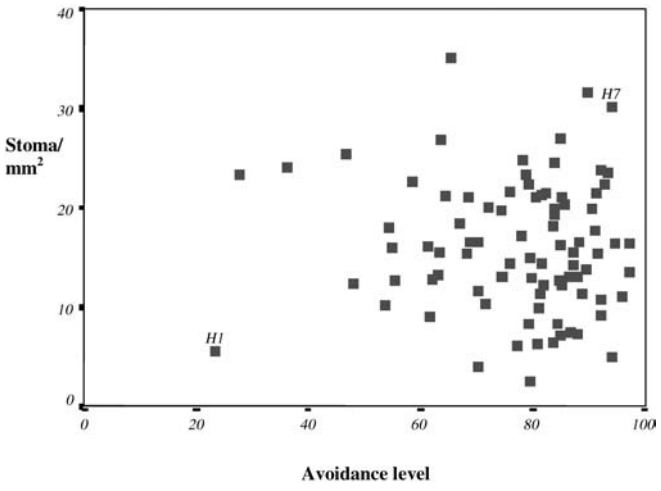


Fig. 2 Avoidance level measured on the adaxial leaf epidermis as the percentage of the germ tube/stoma encounters that did not result in an appressorium and stoma density of the abaxial leaf epidermis on the *H. chilense* F_2 plants from H1×H7 (correlation coefficient = –0.28). The average levels of avoidance and stoma density displayed by the two parental accessions (H1 and H7) are indicated

SEM observations

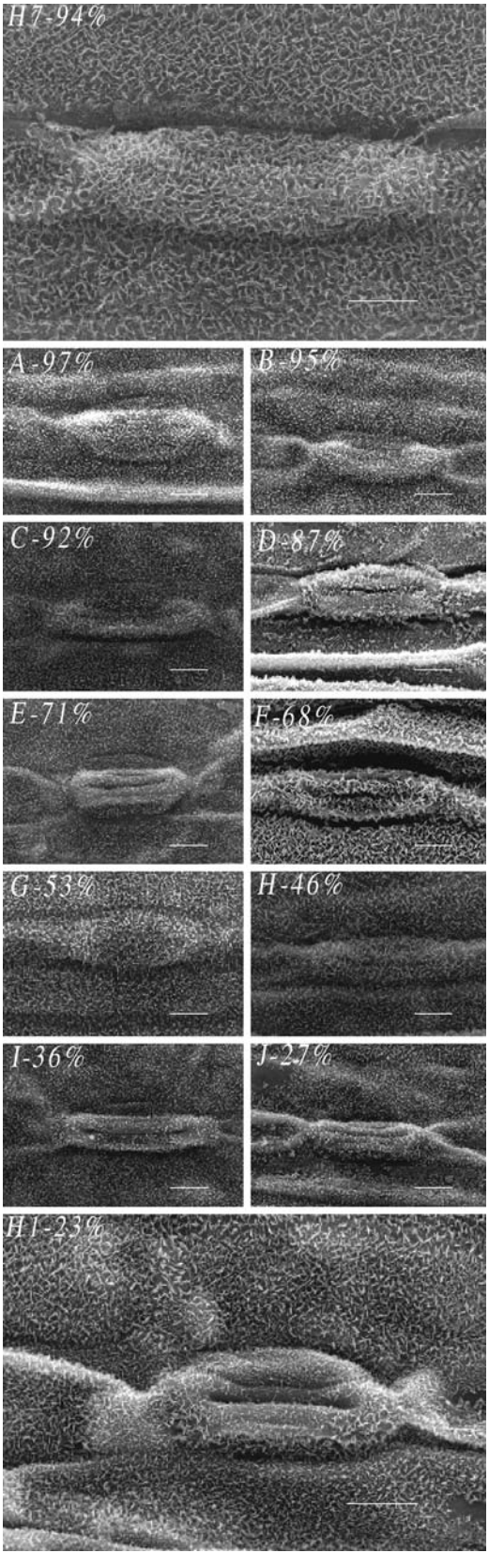
Wax layer differences among *H. chilense* accessions are probably the cause of variation in avoidance level. The contrasting parental accessions and ten F_2 plants, representing the full range of avoidance levels were observed by SEM (Fig. 3). Plants with high avoidance had a more extensive wax covering of the stomata (Fig. 3, H7, 3A, B and C) than plants with a low level of avoidance (Fig. 3I, J and H1). In the high-avoidance plants, the wax covering was so extensive that it was difficult to distinguish the stomata in the epidermis even though they were open. The extent of the wax covering did not vary substantially within leaves. However, in some plants with intermediate levels of avoidance an unexpected level of wax covering of the stoma was found. Some of these plants had stomata quite strongly covered by epicuticular wax (Fig. 3G and H), while other plants had stomata almost completely uncovered (Fig. 3E).

The wax coverage of the stomata in the abaxial epidermis was similar to the wax coverage of the stomata at the adaxial side of the leaves.

All F_2 plants showed vigorous growth, indicating that the variation in wax coverage or in stomata density did not effect the fitness.

Map construction

From the 15 primer combinations selected, a total of 461 distinct AFLP markers were identified and used to construct the linkage map, corresponding to an average of 30 markers per primer combination. Twenty-three markers satisfied the co-dominant genetic criteria (see Materials and methods), and in all cases the molecular



size of the two allelic bands differed by only one or two bases, except for four marker pairs, which differed by 10, 25, 31 and 50 bases. In these markers, the putative heterozygous individuals showed both bands with consistently weaker intensity than the individuals containing only one band. These pairs of AFLP marker bands were considered to be single-locus markers, their names being composed of both allelic fragment sizes, although true allelism should be confirmed by sequence analysis.

The resulting map contained 466 markers (421 dominant and 45 co-dominant), covering a total map distance of 620 cM and corresponding to approximately 1.3 cM per marker (Fig. 4). These markers were assigned to seven long linkage groups and 20 markers to two short groups (shorter than 31 cM) (Fig. 4) when a LOD threshold of 4.0 was chosen for establishing linkage groups. Twenty-three markers were not linked to any linkage group. The nine linkage groups were consistent when using higher LOD threshold values. All of the linkage groups had co-dominant markers and dominant markers from both parental lines.

Inspection of the individual linkage group χ^2 values for goodness-of-fit gives insight into the reliability of the obtained map. The χ^2 values of the majority of the linkage groups were close to 1 (data not shown), except for linkage group 6 (χ^2=2.2) and linkage group 4b (χ^2=1.8). Given the high densities of markers, these χ^2 values indicate that the map is indeed quite reliable.

Sixty-two of the H1-specific AFLP markers were not present in Chinese Spring, but they did occur in one of the addition or substitution lines and hence could serve for assigning a linkage group to a chromosome (Fig. 4). The other H1 markers could not be used since they co-migrated with an amplification product in Chinese Spring or were not clearly visible on the fingerprints of the addition and substitution lines.

Only one of the long linkage groups could not be assigned to one of the seven chromosomes of *H. chilense* since none of the H1 markers on that linkage group were found to occur in any of the addition and substitution lines. This linkage group covered at least 58.0 cM, and could represent either chromosome 3 or the β arm of chromosome 2, since no addition or substitution lines were available for these. However, as a long group had already been assigned to the α arm of chromosome 2, covering a total of 63.2 cM, the length of the linkage group that was not assigned suggests that it probably corresponds to chromosome $3H^{ch}$. Also, the location (group 3TL) of three RFLP markers from other gramineae (Xbcd147-3TL, Xbcd134-3TL and

Fig. 3 Scanning electron microscopy of *H. chilense* adaxial leaf stomatal complexes: *H1* low-avoidance parental accession, *H7* high-avoidance parental accession, **A–J** F_2 plants (H1×H7) representing the range of avoidance levels. Level of avoidance is expressed as the percentage of germ tube/stoma encounters that fail to result in appressorium formation. Notice the extensive wax covering of the guard cells on the high-avoidance parental accession H7

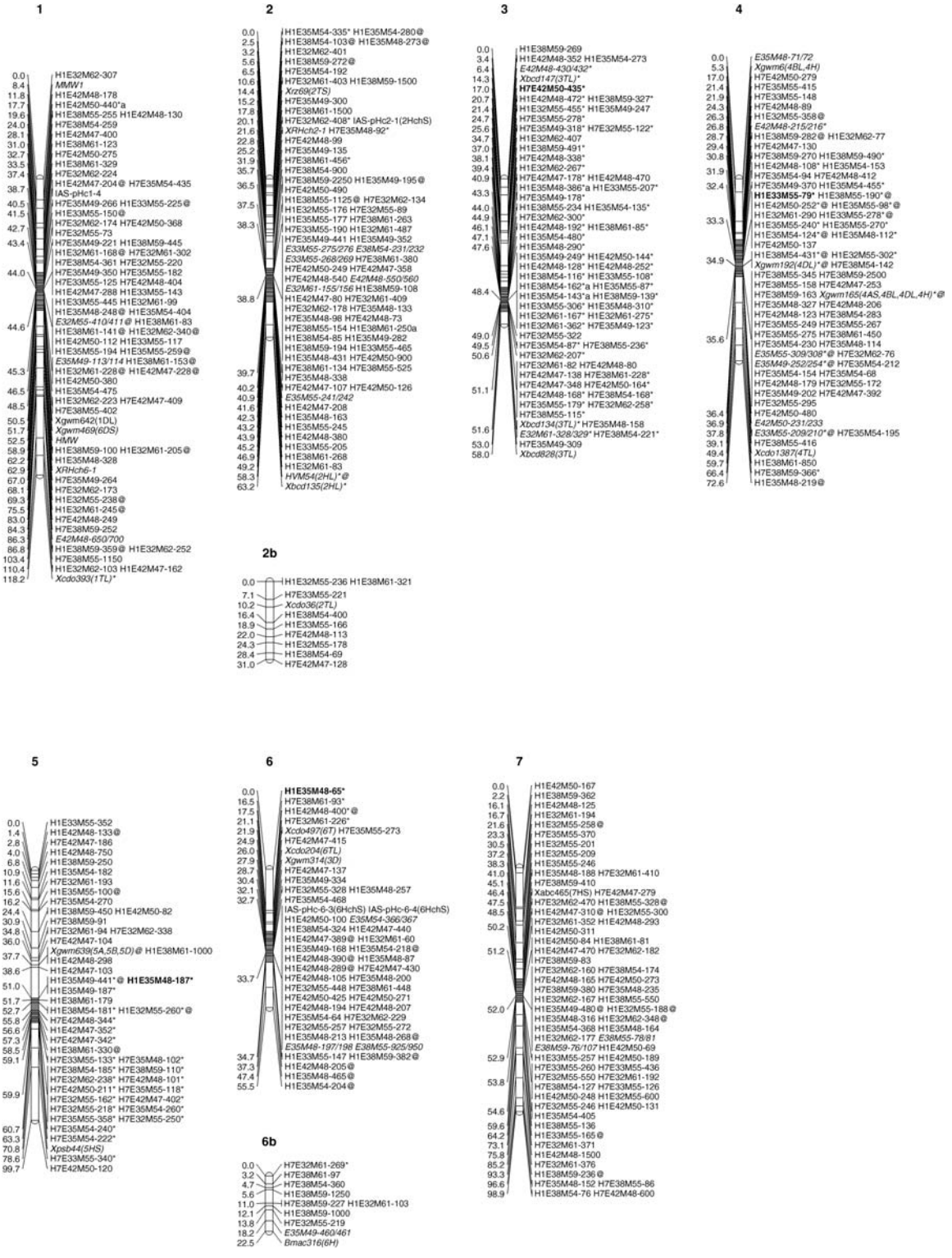


Fig. 4 *H. chilense* linkage map based on an H1×H7 F_2 population. Chromosome-assigned linkage groups are designated *1* through *7*. Lowercase letters indicate multiple linkage groups assigned to the same chromosome but unlinked in this study. Map distances are given in centiMorgans (Kosambi mapping function). @ Markers unambiguously assigned to a specific chromosome by means of the H1 – Chinese Spring substitution and addition lines. a: doubtful amplification products. Co-dominant markers are designated in *italics*. * Markers with skewed segregation ($P \leq 0.005$). Markers with the highest distortion are printed in **bold**. The checkered bars within the chromosomes represent the clusters of markers in the putative centromeric regions. "Anchor markers" (http://greengenes.cit.cornell.edu:80/anchors/) were previously mapped in wheat (A, B or D), barley (H) or in both (T)

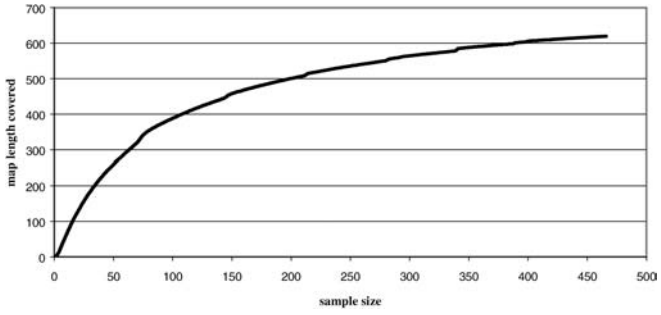


Fig. 5 Relation between number of resampled markers and average map length covered by this sample size. Each data point represents the average over 50,000 replicate samples. A double exponential curve (y = a+b*r**x+c*s**x) was fitted through the data points to estimate the asymptotic value (a)

Xbcd828-3TL) assigned to this linkage group suggests that this linkage group corresponds to $3H^{ch}$ and that there is good colinearity between *H. chilense* and the cultivated Triticeae (Hernández et al. 2001). We will refer to this group as group 3.

Estimation of map coverage of *H. chilense* genome

In order to estimate the proportion of the *H. chilense* genome covered by the present map, we applied the following procedure. From the mapped markers a subset of given size was sampled randomly and the total map length covered by this sample was calculated. For each sample size, running from 1 to 466 (the total number of markers) this was repeated 50,000 times. Through the resulting 'saturation curve' (Fig. 5) a double exponential curve was fitted, resulting in an estimated asymptotic value of 768 cM. Thus, the present map is estimated to cover 620/768 = 81% of the *H. chilense* genome. The validity of the above procedure was extensively tested by using simulated map data, obtained by randomly placing markers, clustered or non-clustered, on a genome of given total length (P. Stam, unpublished).

QTL mapping

QTLs controlling avoidance

With a LOD threshold of 3.7, two QTLs for the avoidance trait, on two different chromosomes, were detected in this F_2 population and designated *qavoi1* and *qavoi2* (Table 1, Fig. 6). The QTL with the largest effect, *qavoi2*, was located on chromosome 5 and explained about 24% of the phenotypic variance. Together, the two QTLs explained 40% of the phenotypic variance. For the two QTLs, H7 alleles increased the level of avoidance, which is in agreement with the lack of transgressive segregation. Additive effects of the H7 alleles for *qavoi1* and *qavoi2* were +5.5 and +9.7% germ tube/stoma encounters that do not result in appressorium formation, respectively. Using the estimated additive effects of the H7 alleles on the two QTLs, we predicted a difference of 30.4% in avoidance level between the two parental accessions H1 and H7, based on a simple additive model (double the sum of the additive effects). The observed difference between the two parental accessions H1 and H7 was of 71% of germ tube/stoma encounters without appressorium differentiation (Fig. 1a). This implies that the detected QTLs explain only 43% of the phenotypic difference between the parental accessions, indicating that not all the QTLs controlling avoidance were detected with this experiment.

There was an indication, below the LOD threshold (3.7), for the presence of two additional minor QTLs controlling avoidance on chromosome 1 and on chromosome 6. The suggested QTL located on chromosome 1 explained about 12% of the phenotypic variance and had a peak LOD score of 2.99 on the marker H1E32M61-205. The suggested QTL on chromosome 6 had a peak LOD score on the marker H1E42M48-205 of 3.15 and explained 10% of the phenotypic variance.

QTLs controlling stoma density

With a 3.4 LOD threshold level, three QTLs were identified for stoma density (Table 1, Fig. 6). The three QTLs (*qstod1*, *qstod2* and *qstod3*) explained together 63% of the phenotypic variation and were located on chromosomes 1, 3 and 7. Two alleles for increasing and one for decreasing stoma density were contributed by H7 (additive effect of –12.1, +13.0, and +10.4 stomata/mm^2, respectively). However, transgressive segregation was not observed in the F_2 population. Some F_2 plants had an average stomata density higher than H7 or lower than H1, but these differences were not significant. Using a lower LOD threshold, we were able to detect an extra QTL for stoma density with minor effect on chromosome 6. The suggested QTL explained about 6% of the phenotypic variance and had a peak LOD score (3.3) on the marker H1E38M59-1000.

Chromosome 3 included QTLs for the avoidance trait and for stoma density. There was overlap of the supporting confidence intervals, suggesting that these QTLs were tightly linked.

Additive effects of QTLs

Multiple QTL mapping, as implemented in MAPQTL, assumes that QTLs have only additive effects and no epistatic effects (Jansen 1993). By applying a two- and a three-factor (two or three significant QTLs for each trait) analysis of variance based on the genotype values of the 'peak' markers of each QTL, we tested whether indeed the QTL effects are additive or whether significant interactions occur between them. No significant interactions among QTLs were detected for either of the two traits (Tables 2 and 3).

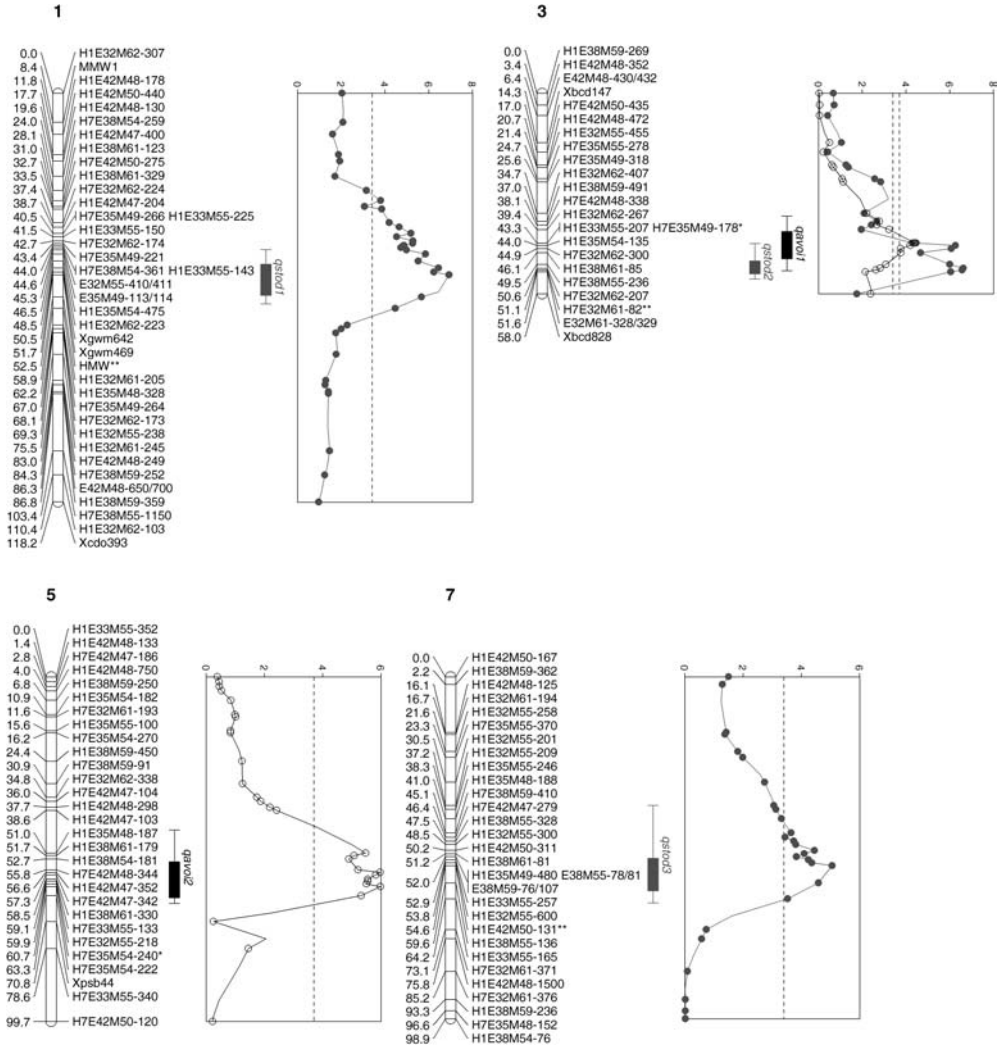


Fig. 6 Genetic linkage map of *H. chilense* chromosomes 1, 3, 5 and 7 showing the positions of QTLs for avoidance level and stoma density. The map was developed using the F_2 population H1×H7. Map positions are given in centiMorgans, using the Kosambi function. AFLP marker loci were assigned the prefix *H1* or *H7*, encoding the parental line that produced the amplified DNA fragment. *Bars* to the right of the chromosome maps indicate the QTL locations with a two LOD support confidence interval (*empty circle* avoidance level, *solid circle* stoma density). QTL likelihood profiles for restricted multiple QTL model are depicted with the LOD significance threshold values as a *dotted line* (3.7 for avoidance level and 3.4 for the stoma density). Markers used as co-factors are indicated by * and ** for avoidance level and stoma density, respectively

Table 1 Quantitative trait loci for avoidance level and stoma density on the abaxial leaf epidermis in an *Hordeum chilense* F_2 (H1×H7)

QTLs	Co-factor marker	Chromosome	Interval length[a] (cM)	QTL position (cM)	Additive effect[b]	Peak LOD score	Percentage variance explained
Avoidance							
qavoi1	H7E35M49-178	3	16.0	43.3	+5.5	4.4	16.8
qavoi2	H7E35M54-240	5	21.0	60.7	+9.7	6.0	23.6
Stoma density							
qstod1	HMW1	1	14.0	52.5	–12.1	6.9	23.1
qstod2	H7E32M61-82	3	10.0	51.1	+13.0	6.6	22.8
qstod3	H1E42M50-131	7	28.0	54.6	+10.4	5.1	16.9

[a] The support interval was estimated at a LOD fall off –2.00
[b] (Mean of the H7 allele genotypes – mean of the H1 allele genotypes)/2. Avoidance measured as the percentage of the germ tube/stoma encounters that does not result in an appressorium and stoma density as stomata/mm^2

Table 2 Two-factor analysis of variance for avoidance level

Source of variation	Mean square	*F*	Significance
H7E35M49-178	0.3260	19.268	0.000
H7E35M54-240	0.4030	23.838	0.000
H7E35M49-178* H7E35M54-240	0.0004	0.024	0.878
Error	0.0169		

Table 3 Three-factor analysis of variance for stoma density

Source of variation	Mean square	*F*	Significance
HMW1	95.314	3.181	0.080
H7E32M61-82	168.184	5.613	0.022
H1E42M50-131	203.667	6.797	0.012
HMW1* H7E32M61-82	37.993	1.268	0.265
HMW1* H1E42M50-131	8.803	0.294	0.590
H7E32M61-82* H1E42M50-131	10.022	0.334	0.565
HMW1* H7E32M61-82* H1E42M50-131	37.461	1.250	0.269
Error	29.965		

Discussion

A high association between avoidance of rust fungi and stoma density on the abaxial leaf epidermis of *H. chilense* had already been described by Rubiales and Niks (1996). The present study was undertaken to determine the nature of this association. Rubiales and Niks (1996) suggested that the association between both traits was either due to pleiotropy, to close genetic linkage between genes for avoidance and for stoma density or to the linked evolution of both traits over ecotypes. The insignificant correlation between avoidance level and stoma density on the abaxial leaf (r = –0.28, Fig. 2) and the lack of co-localisation of the QTL regions for avoidance and stoma density (Fig. 6) rule out the possibility of pleiotropy and close genetic linkage, and therefore support the conclusion by Vaz Patto et al. (2001) that avoidance and stoma density are associated between particular ecotypes of. *H. chilense*.

QTL analysis revealed that the genetic control of avoidance and stoma density was indeed polygenic. Two QTLs for the avoidance of barley leaf rust and three QTLs for stoma density were detected. The number of detected QTLs may have been underestimated as QTLs may have escaped detection because of the scarcity of markers in some map regions, the small population size (100 F_2 individuals) and a relatively high LOD threshold (3.7 and 3.4) used to reduce the rate of false positives (Young 1996). Even so, the detected QTLs could explain 40% of the phenotypic variation for avoidance level and 63% of the phenotypic variation for stoma density.

In the interaction *H. chilense-P. hordei*, extensive wax coverage over the guard cells, obscuring the features that normally induce appressorium differentiation, was suggested to be the cause of overgrowth of stomata in *H. chilense* accessions with high avoidance levels (Rubiales and Niks 1996). The role of the epicuticular wax layer coverage on avoidance was confirmed by Vaz Patto and Niks (2001). On high-avoidance accessions, the removal of the wax layer allowed appressoria to develop over stomata that would otherwise be overgrown. Removal of the wax layer on the low-avoidance accessions did not significantly affect appressorium differentiation.

Avoidance of rust fungi in wild barley, *H. chilense*, occurs for at least four different leaf rust species (Rubiales and Niks, 1992a, 1996). The level of avoidance of 37 *H. chilense* accessions to barley leaf rust (*P. hordei*) and to wheat leaf rust (*P. triticina*) was very highly correlated (r = 0.97; Rubiales and Niks 1996). Both species of rust fungus show appressorium differentiation response to similar patterns of topographical stimuli on artificial membranes (Collins and Read 1997). This explains why the avoidance effective against *P. hordei* is also effective against *P. triticina*.

In the present study, SEM on F_2 plants confirmed that plants with a high avoidance level showed a more extensive wax coverage of the stomatal complexes than plants with a low level of avoidance (Fig. 3). However, on plants with intermediate levels of avoidance this association was not so obvious. A greater variation in wax coverage among the intermediate phenotypes than among the most extreme phenotypes for avoidance was observed. This could suggest that the wax covering of stomata is not the only factor determining the level of avoidance of these plants.

As reported by Farquhar and Richards (1984) the ratio $^{13}C/^{12}C$ correlates positively with water use efficiency and negatively with stomatal conductance. Rubiales and Niks (1996) did not find significant differences for this $^{13}C/^{12}C$ ratio between avoidance and non-avoidance accessions, concluding that the extensive wax covering of *H. chilense* avoidance accessions does not decrease stomatal conductance. However, no stoma density was taken into account in their measurements. The authors compared water use efficiency per leaf, rather than per stoma. We suggest that the extensive wax covering of the stomata on the avoidance accessions may reduce stomatal conductance but that the higher stoma density on the abaxial epidermis may compensate for this, resulting in a similar water use efficiency as in low-avoidance accessions.

No epistatic effects were found between the detected QTLs for avoidance. The QTLs identified had only additive effects. This may imply that the inheritance of this trait is not particularly complex, opening the way for relatively straightforward use of markers in breeding for avoidance. Breeding for resistance to pathogens is an important aspect of plant breeding programmes. The knowledge of the genetic basis and location of the QTLs responsible for the avoidance character will facilitate the transfer of this trait from *H. chilense* to the genomes of related cultivated species.

Rubiales et al. (1991) showed that, unfortunately, rust avoidance of line *H. chilense* H7 is not expressed in tritordeum, the allohexaploid hydrid between *H. chilense* and tetraploid wheat. This suggests that the genes for

avoidance in *H. chilense* are not expressed in a wheat genomic background, at least not if the complete genome of *H. chilense* is present.

Molecular markers could be used to support the introgression of the chromosomal regions harbouring the loci that confer the avoidance in *H. chilense* to wheat leaf rust into wheat. Disomic addition wheat lines based on avoidance line H7 of *H. chilense* will show whether the avoidance character is expressed in a wheat genomic background to with only one or two *H. chilense* chromosomes have been added.

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