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Linkage disequilibrium mapping of morphological, resistance, and other agronomically relevant traits in modern spring barley cultivars

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Abstract

A set of 148 modern spring barley cultivars was explored for the extent of linkage disequilibrium (LD) between genes governing traits and nearby marker alleles. Associations of agronomically relevant traits (days to heading, plant height), resistance traits (leaf rust, barley yellow dwarf virus (BYD)), and morphological traits (rachilla hair length, lodicule size) with AFLP markers and SSR markers were found. Known major genes and QTLs were confirmed, but also new putative QTLs were found. The LD mapping clearly indicated the common occurrence of *Rph3*, a gene for hypersensitivity resistance against *Puccinia hordei*, and also confirmed the QTL *Rphq2* for prolonging latency period of *P. hordei* in seedlings. We also found strong indication for a hitherto not reported gene for resistance or tolerance to BYD on chromosome 2, linked to SSR marker HVM054. Our conclusion is that LD mapping is a valuable additional tool in the search for applicable marker associations with major genes and QTLs.

Introduction

Marker assisted selection has been considered as a promising application of molecular genetics (Young 1999; Dekkers and Hospital 2002), that would greatly enhance the effectiveness of plant breeding programs. An important step is finding molecular markers that are located close to genes that govern the trait phenotype of interest. In most crops molecular marker maps have become available, and many interesting genes have been mapped, including many quantitative trait loci (QTLs). Typically, for QTL mapping, two contrasting genotypes are crossed, e.g. two inbred lines, and the association between mapped marker loci and phenotypic traits allows the identification of QTLs on the genome. Recently, instead of bi-parental crosses, collections of cultivars, lines, or landraces are used to identify marker – trait associations in rice (Virk et al. 1996), oat (Beer et al. 1997), and barley (Igartua et al. 1999; Kraakman et al. 2004). This methodology has become popular in human genetics under names as association mapping or linkage disequilibrium (LD) mapping, and led to a number of successes (see for a review Cardon and Bell 2001). In plant genetics, using a collection of cultivars has a number of advantages over the use of a bi-parental cross. Firstly, in the population a broader genetic variation in a more representative genetic background will be available. This implies that one is not limited to the marker and trait loci that happen to differ between two parents.

Secondly, LD mapping may attain a higher resolution, because of the use of all meioses accumulated in the breeding history. Thirdly, historic phenotypic data on cultivars can be used to link markers to traits, without the need for new trials with special mapping populations. The methodology for associating markers and traits in a collection of cultivars are still under development. Some basic issues were addressed by Jannink and Walsh (2002).

In order to be able to identify marker – trait associations, LD has to occur in the germplasm. LD will tend to decay with genetic distance between the loci under consideration, because genetically distant loci are more likely to have recombined in the past than tightly linked loci. In populations, for any pair of linked polymorphic loci LD decreases over generations, because of accumulation of recombination. Finally the loci will be in linkage equilibrium (LE), i.e. alleles are not preferentially paired anymore. LD may also occur in a population due to selection, for instance when an important trait is regulated by multiple loci, or due to recent introductions of genotypes. The process of decrease of LD to reach LE depends on the opportunities of genetic recombination between the allele pairs of the loci under consideration. For effective recombination double heterozygotes are required, and these are much more common in allogamous than in autogamous plant species. Therefore, LD will tend to be more obvious after repeated inbreeding, as in autogamous species, than in outcrossing species. In several crops LD has been studied and quantified. In sugar beet, LD extended up to 3 cM (Kraft et al. 2000), while in some *Arabidopsis* populations LD exceeded even 50 cM (Nordborg et al. 2002). Kraakman et al. (2004) showed that in a set of 148 modern spring barley cultivars markers at distances up to 10 cM tend to be in LD (see their Figure 2).

The focus of the present article is on LD mapping in the same set of 148 modern spring barley cultivars for phenological, resistance, and morphological traits. The traits were days to heading, plant height, leaf rust resistance, barley yellow dwarf (BYD) resistance, rachilla hair length and lodicule size. Positions of markers that showed high association with trait values were compared with those of loci known to govern the trait. We used pedigree information to understand whether certain cases of LD between a marker and a trait might be explained by particular ancestors of modern cultivars.

Materials and methods

Materials

A total of 148 modern European two-row spring barley cultivars were used, representing commercial germplasm used all over north-west Europe in the past 15 years (see Kraakman et al. 2004 for more details). The list of cultivars and the breeding companies that bred them are presented as supplementary data.

The barley cultivars were exposed to leaf rust (*Puccinia hordei*) isolate 'IVP2000', which is virulent against the hypersensitivity genes *Rph9* and *12*, and avirulent against the commonly present *Rph3* gene (Niks et al. 2000). Only cultivars without effective hypersensitivity to 'IVP2000' provided the data on the basis of which the level of partial resistance was assessed. Partial resistance is a resistance that is not based on a hypersensitivity reaction, but causes a reduced rate of development of uredia and a reduction in their number (Parlevliet 1975; Niks et al. 2000). This resistance against the leaf rust is controlled by few to many genes with additive effects (e.g. Qi et al. 1998b).

BYD occurred by natural infection. The experiment was adjacent to the 2001 verification trial as reported by Niks et al. (2004), and therefore probably due to the same viral strain(s) as in that trial.

Seedling tests for barley leaf rust: infection type and latency period

Seedlings were raised in 37×39 cm plant boxes. Each box contained 6 or 7 cultivars, and L94 and Vada as references. Per cultivar two to five seedlings were available. About 10 days after sowing the first leaves were fixed in horizontal position and inoculated in a settling tower. Each box received 3.5 mg inoculum, which amounts to about 200 spores per cm^2. About ten times as much volume of Lycopodium powder was added to the inoculum to obtain a homogeneous

distribution of the inoculum over the plant boxes. After incubation at a relative humidity of 100% overnight, the seedlings were transferred to a greenhouse.

Infection type (IT) was determined on the seedling leaves using the scale of 0–9 described by McNeal et al. (1971). This scale takes into account degrees of necrosis, chlorosis and sporulation associated with the infection sites. IT0: no symptoms; IT1 and IT2: minute or small necrotic/chlorotic flecks, respectively, no sporulation; IT3, 4, 5, 6: trace to much sporulation, respectively, and pustules surrounded by clear and serious necrosis and many flecks without sporulation (IT3) to clear chlorosis around the pustules and few flecks without sporulation (IT6); IT 7, 8 and 9: abundant sporulation, with some (IT7) to no chlorosis at all (IT9).

The latency period (LP) of seedlings was evaluated by estimating the period (hours) at which 50% of the ultimate number of pustules became visible. The relative latency period (RLP) was calculated relative to the LP measured on L94 seedlings, where the LP on L94 = 100, as described by Parlevliet (1975). RLP is a component of partial resistance that is conveniently estimated in a monocyclic experiment in a greenhouse compartment. RLP was measured in two consecutive experiments in 2001 and 2003. We estimated the genotypic means for RLP from the fit of an additive model (phenotype = genotype + trial).

Field tests: AUDPC, BYD, PH, and DTH

In 2001, the cultivars were planted in a randomized block design with two replications, where the second replication contained fewer cultivars because of seed limitations. The plot size was 0.75×1.25 m^2. In each strip of 14 plots, the reference cultivar L94 was represented as one of the plots. The plots of barley lines alternated with plots of oats to limit the inter-plot interference. One month after sowing, more than 350 young L94 plants, raised in pots, were inoculated with leaf rust isolate IVP2000 in the greenhouse. One week later, the sporulating L94 plants were placed in the alleyways between the field plots in front of each barley plot. Approximately 10 days later, when the plants in the L94 reference plots started to sporulate, the spreader plants were removed.

The amount of leaf rust infection in the field was determined three times on the upper three leaves of three tillers per plot. The leaf rust pustule counts were transformed according to the scale proposed by Parlevliet and Van Ommeren (1984). The Area Under the Disease Progress Curve (AUDPC) was calculated and used as trait for linkage disequilibrium mapping. Furthermore, days to heading (DTH) was evaluated as the number of days from sowing until 50% of the plants in the plot had headed. Plant height (PH) was measured as the number of centimeters between ground and the basis of the ear, averaged for eight plants in the final stage of plant development. BYD occurred by natural infection. BYD tolerance was assessed on adult plants on June 28, July 1st, and July 4th. The severity of symptom development was scored on a 0–5 scale. The score took into account both the proportion of the plot that was affected and the severity of the symptom development per plant. A detailed description of the different levels of scoring can be found in Niks et al. (2004).

For all traits, we estimated their genotypic means from the fit of an additive model (phenotype = genotype + trial). These genotypic means were used for the assessment of associations between traits and markers.

Seeds: rachilla hair length and lodicule size

The observations of both rachilla hair length and lodicule size were done on at least two grains per accession. The rachilla was evaluated directly under binocular preparation microscope, and the size of the lodicules after removing the basal part of the lemma. Rachilla was rated as short (0) or long (1), and lodicules as small (0) intermediate (1) or large (2).

Genotyping and map construction

The cultivars were genotyped with 14 AFLP-primer combinations and 11 microsatellites (SSRs). The AFLPs were run as described by Qi and Lindhout (1997) and Qi et al. (1998a), and resulted in 286 polymorphic markers. For analyses, 236 markers with band frequencies in

between 5% and 95% were used. For 123 markers the map position could be established, using an integrated map of three segregating populations (see for details Kraakman et al. 2004). The integrated map is available through http://www.dpw.wau.nl/pv/, and also presented in Figure 2. Five extra AFLPs and 11 SSRs were added to the integrated map on the basis of their significant association with AFLP markers on our integrated map. The position of AFLPs could be confirmed with information from other mapping populations like L94×116–5 (Qi et al. 2000) and L94×C123 (unpublished). The SSRs we used were Bmac018, Bmag009, HVM14, HVM22, HVM65, HVM74, Bmag223, Bmac134, HVM054, Bmac163, and Bmac316. The selection of SSRs was based on their map position according to Ramsay et al. (2000). We selected SSRs in regions where the integrated AFLP-map showed gaps, especially when in those regions interesting genes might be located. For instance, the first six SSRs mentioned above were selected in the neighborhood of the leaf rust QTL *Rphq3* on chromosome 6. The protocols used were according to Macaulay et al. (2001) and Ramsay et al. (2000). The electrophoreses were run on a DNA sequencer 4200 (LI-COR) under 1500 Volt, 40 mA, 45 °C buffer temperature, and 25 Watt power. The data were collected with a single scanning laser on two infra-red frequencies, and automatically saved to a computer image.

Linkage disequilibrium mapping and statistical analysis

The simplest test for association between a binary marker like AFLP, for which either band presence or absence is scored, and a quantitative trait, is a two-sample *t*-test, where the presence of the band defines one sample and the absence of the band defines another sample. When the average of the cultivars with the band differed significantly from the average of the cultivars without the band, we interpreted this as evidence for the existence of a marker-trait association. The test statistic in this case is the difference in mean between cultivars with and without band divided by the standard error of this difference (for details, see, for example, Snedecor and Cochran 1980). From a computational point of view, it was easier to calculate a marker-trait association for an AFLP as a Pearson correlation coefficient between a phenotypic response vector containing the trait values and another vector representing the AFLP marker that carried the value 1 whenever a band occurred and zero otherwise. This is effectively equivalent to a *t*-test using marker incidence as grouping variable. Under the null hypothesis of no marker-trait association and the assumption that the error follows a normal distribution with zero mean and constant variance, the test statistic for a Pearson correlation, $t^* = r\cdot(n-2)^{1/2}/(1-r^2)^{1/2}$, with r the correlation and n the total number of observations (cultivars), follows a $t_{(n-2)}$ distribution. For some traits, the assumption of normality for the error was unrealistic and a non parametric alternative to the *t*-test was used. Thus, for the trait IT, we used the Spearman rank correlation instead of the Pearson correlation. For the multi-allelic SSRs, we treated the individual SSR alleles as separate indicator variables, that took the value 1 whenever a (homozygous) cultivar carried the allele and 0 otherwise. To assess the association of each SSR marker locus with traits, we fitted a multiple regression model of the trait response on the set of allele indicator variables. The corresponding R^2 and *p*-value were used for further analyses.

To control for multiple testing, we used a procedure that estimates the false discovery rate (FDR) (Benjamini and Hochberg 1995). The FDR is the proportion of false positive tests among the significant tests, or, the proportion of marker-trait associations that were declared significant, although in reality no association existed. FDR for individual tests can be expressed as a *q*-value that represents the expected proportion of false positives incurred when calling the observed value for the test-statistic significant. FDR *q*-values can be used alongside the commonly used *p*-values. FDR can be useful in genome wide studies where often many markers are tested for association with many traits (Weller et al. 1998; Storey and Tibshirani 2003; Kraakman et al. 2004). Storey and Tibshirani (2003) adapted a version of FDR that takes into account the density distribution of realized *p*-values to estimate the FDR for a batch of tests. We used their software called QVALUE, with

default settings (π_0-method = smoother, FDR level = 0.05). This software can be downloaded at www.genomine.org/qvalue.

Population structure

Population structure can result in false associations, and should therefore be prevented or taken into consideration if it occurs. To investigate possible structure in the set of cultivars we performed three analyses. First, a cluster analysis, second a correspondence analysis, and finally an analysis based on a Bayesian model (Pritchard et al. 2000). Details about these analyses can be found in Kraakman et al. (2004).

Results

Assessment of resistance and plant development traits

The set of cultivars showed variation for all traits observed (Table 1). The IT of leaf rust infection was for 33 cultivars low (≤ 6; hypersensitive reaction) and for 113 cultivars high (>6; compatible reaction). The rachilla hair length was short for 17 cultivars and long for 131 cultivars. The lodicule size was small/intermediate/large for 33/13/101 cultivars, respectively.

IT and AUDPC were significantly and positively correlated: effective hypersensitivity, expressed as low IT, resulted in low AUDPC scores in the field (Figure 1, Table 2). Also significantly correlated were AUDPC with RLP, DTH with PH, and rachilla hair length with BYD score.

Cultivars with an IT >6 had an AUDPC higher than 166, and cultivars with an IT≤ 6 had an AUDPC lower than 160 (Figure 1). In the latter group was one exception, Hanka, which had a high AUDPC (215), despite a hypersensitive reaction (IT = 4) in the seedling stage.

AFLPs and SSRs

Information on the AFLPs used can be found in Kraakman et al. (2004). In this study, the set of molecular markers was extended with 11 SSRs. The number of alleles found per SSR ranged from two to seven. For those cultivars in our set that had also been genotyped for SSR markers by other authors (Ramsay et al. 2000; Russell et al. 2000; Macaulay et al. 2001), the size of amplification products was in agreement with those reports. The position of the 128 AFLP and 11 SSR markers on the integrated map is presented in Figure 2.

Population structure

To investigate population structure, we performed three types of analysis. Various analyses using the Bayesian clustering methodology described in Pritchard et al. (2000) did not suggest a distinct subpopulation structure. However, hierarchical cluster analysis as well as correspondence analysis hinted at the existence of two sub-groups (see Kraakman et al. 2004). This split

Table 1. Descriptive statistics for Infection Type (IT), Relative Latency Period (RLP), and AUDPC for leaf rust resistance, and Barley Yellow Dwarf Virus tolerance (BYD), Days to Heading (DTH), and Plant Height (PH) of 148 barley cultivars. DTH is given in days, PH in cm.

Trait	*N*	Minimum	Percentiles					Maximum
			5%	25%	50%	75%	95%	
IT	146	1	2	9	9	9	9	9
AUDPC	140	69.9	101.0	189.4	223.2	234.6	245.8	258.7
AUDPC IT > 6	107	166.2	194.7	217.5	229.8	236.5	247.2	258.7
RLP	111	101.6	104.3	107.4	109.5	113.0	118.0	124.0
BYD	140	0.33	0.52	1.99	2.67	3.00	3.50	4.00
DTH	140	51.0	52.0	53.0	54.5	56.5	60.0	64.0
PH	140	63.8	66.4	72.2	76.3	79.5	84.2	93.1

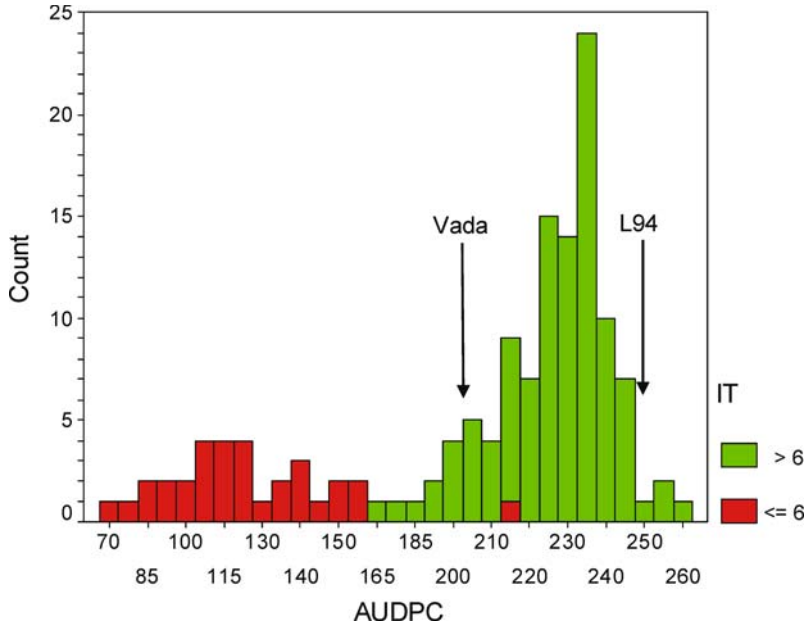

Figure 1. Frequency distribution of phenotypes for AUDPC of 148 barley cultivars, exposed to *Puccinia hordei*. Values of Vada (202.2) and L94 (247.2) are shown by arrows.

Table 2. Correlations between trait values in 148 spring barley cultivars. For IT Spearman rank correlation is given, for all other traits Pearson correlation coefficient. Significance *, **, *****, $p < 0.01$, 0.001, 0.000001, respectively.

Trait	IT	AUDPC	AUDPC IT > 6	RLP	BYD	Heading	Height	Rachilla length
IT								
AUDPC	0.70*****							
AUDPC IT > 6	−0.07	1.00						
RLP	−0.13	−0.29*	−0.32*					
BYD	0.04	0.07	0.12	−0.25				
DTH	0.13	0.14	−0.05	0.08	−0.02			
PH	−0.04	−0.01	0.09	−0.06	−0.12	−0.22*		
Rachilla length	0.00	0.02	0.00	−0.23	0.31**	0.06	0.00	
Lodicule size	−0.08	−0.01	0.25	−0.12	−0.09	−0.10	0.14	−0.05

in the germplasm could not be explained by geographic arguments, or by a separation of fodder and malting barleys.

Overview of all associations

All significant marker-trait associations are shown in Table 3, for both mapped and unmapped markers. Furthermore, the mapped associated markers are graphically shown in Figure 3 with relevant QTLs reported in literature. For all traits the most significant marker-trait associations will be discussed.

Linkage disequilibrium mapping of leaf rust resistance

IT: Rph3 confirmed

Marker E39M61–255 on chromosome 1 at 151 cM was highly associated with IT of *P. hordei* inoculated seedlings (Spearman's $r = 0.74$; $p < 1E\text{-}6$) (Table 3). In this genome region, a major gene for leaf rust resistance, *Rph3*, has been identified (Chelkowski et al. 2003). As the marker-trait association of this marker was very high in this set of cultivars, we can conclude that *Rph3* was responsible for most of the variation in IT. Resistance to leaf rust (= low IT) corresponded to

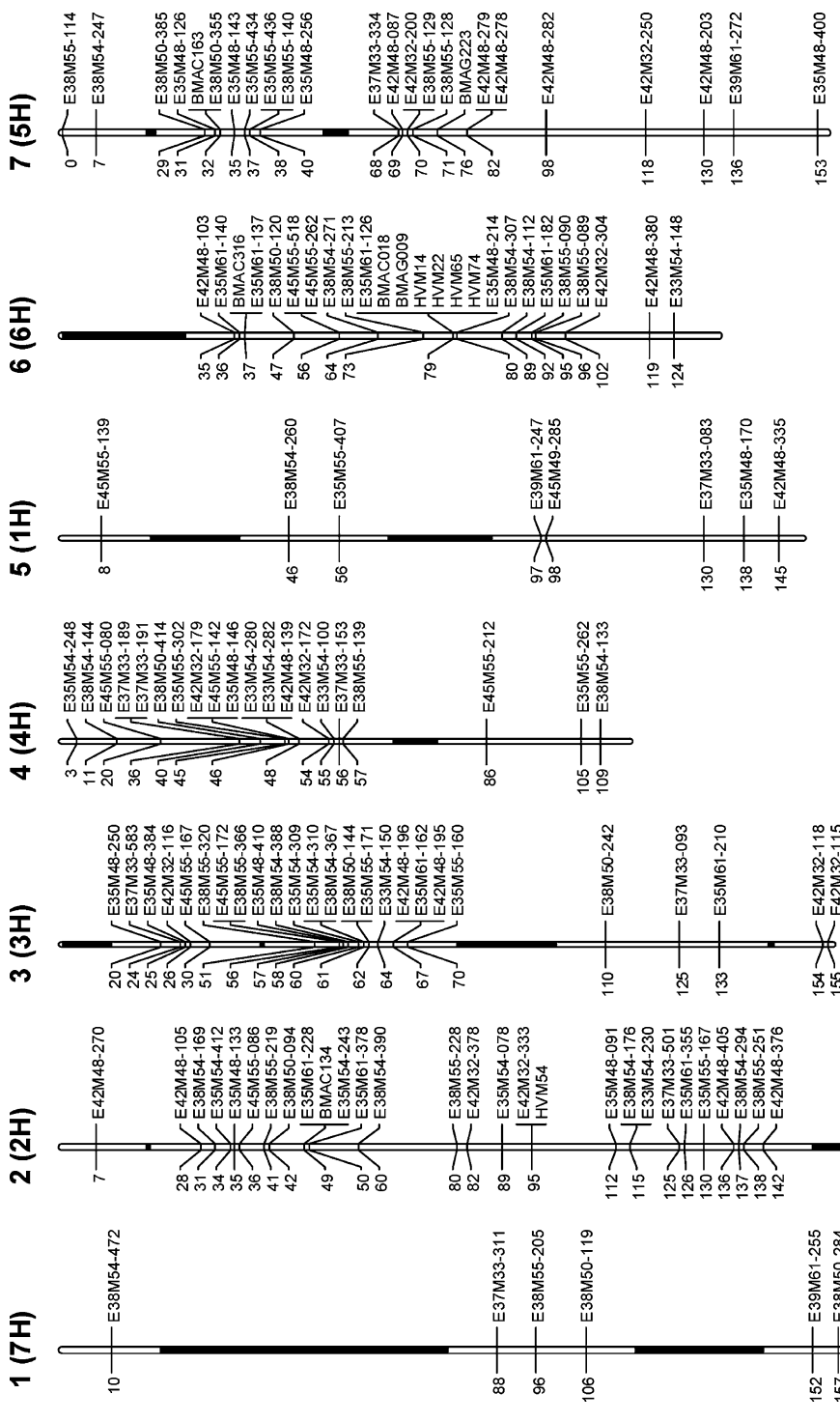

Figure 2. Integrated map showing the genetic position of 128 AFLP markers and 11 SSR markers that were used to detect association with the traits. Assuming that linkage disequilibrium occurs up to distances of 10 cM, trait loci in areas more than 10 cM away from any marker, highlighted in black, were unlikely to be in LD with these mapped markers.

Table 3. All marker-trait associations found in 148 barley cultivars for markers where at least one association (printed bold) had a false discovery rate (FDR) of less than 5% ($q < 0.05$) (see Material and methods).

No.	Marker	chr	pos[1]	AUDPC high IT				RLP[4]				IT				BYD				DTH				PH			
					r	*p*[3]	Known[2]		*r*	*p*[3]	Known[2]		*r*	*p*[3]	Known[2]		*r*	*p*[3]	Known[2]		*r*	*p*[3]	Known[2]		*r*	*p*[3]	Known[2]
1	E38M54-472	1	9		−0.17				0.27	*			−0.13			1	**−0.48**	****	d		0.07				0.01		
2	*E42M32-231*	*1*	*24*		0.01				0.03				−0.02				0.05				0.00			1	**−0.27**	*	j,g
3	E38M50-119	1	106		0.04				−0.24				0.05				−0.18				0.08			2	**0.30**	**	f,g,e
4	E39M61-255	1	151		−0.15				−0.07				**0.74**	****	h,i		0.04				0.10				−0.04		
5	E35M54-183	1	163		−0.04				−0.03				−0.06			2	**−0.28**	**	c		−0.16				0.12		
6	E35M61-228	2	49		0.25			1	**−0.34**	*	a,e		0.05			3	**0.47**	****	d		−0.02				0.05		
7	E35M54-078	2	89		0.04			2	**0.34**	**	a		−0.18				−0.12				0.12				−0.22	*	
8	HVM054	2	95		0.32				0.37				0.22			4	**0.67**	****			0.25				0.20		
9	E42M48-405	2	136		−0.22			3	**0.33**	**	a,e,k		0.18				0.15				0.00				−0.11		
10	E38M54-294	2	137		0.04			3	−0.10		a,e,k		**−0.29**	**			0.10				0.02				0.15		
11	E38M55-251	2	138		−0.22			3	**0.42**	****	a,e,k		0.22				−0.06				−0.03				−0.10		
12	E42M48-376	2	142		−0.21			3	**0.37**	***	a,e,k		**0.28**	**			−0.03				−0.08				−0.15		
13	E45M55-172	3	56		−0.06			4	**0.38**	***			−0.03				−0.07				−0.04				0.03		
14	E35M54-310	3	61		0.07			4	**−0.37**	***			0.06				0.08				0.05				−0.03		
15	*E45M55-154*	*3*	*76*		−0.03			4	**−0.35**	**			0.18				0.17				0.01				0.14		
16	*E45M49-164*	*4*	*0*		0.07				0.10				0.21			5	**0.34**	**			0.06				−0.21		
17	E35M55-302	4	45		0.19				−0.15				−0.03			6	**0.25**	*			−0.08				−0.07		
18	E38M50-355	7	32		−0.18				0.18				−0.14				−0.12			1	**−0.29**	*	l,m		0.16		
19	E42M48-087	7	69	1	**−0.29**	*			0.06				−0.11				0.02				0.22	*			−0.19		
20	E42M32-200	7	70	1	**−0.38**	***			0.25				0.14				0.14				0.04				0.00		
21	E38M55-128	7	71	1	**−0.38**	***			0.02				0.06				0.18				−0.04				−0.12		
22	BMAG223	7	76	1	0.26				0.10				0.20				0.22				0.32			3	**0.37**	*	j,l
23	E42M48-279	7	82	1	**−0.30**	*			0.10				0.02				−0.02				−0.06				−0.04		
24	E33M54-063	unm.			−0.07				**0.36**	**			−0.03				−0.08				−0.03				−0.01		
25	E33M54-095	unm.			−0.04				−0.03				−0.03				0.19				**−0.28**	**			0.00		
26	E33M54-421	unm.			0.24				−0.19				−0.01				**0.29**	*			0.10				−0.06		
27	E35M48-095	unm.			0.03				−0.13				−0.08				−0.01				−0.14				**0.28**	**	
28	E35M48-111	unm.			−0.02				−0.08				−0.09				0.08				−0.11				**0.33**	***	
29	E35M48-233	unm.			0.12				−0.01				0.12				0.01				−0.11				**0.29**	*	
30	E35M54-147	unm.			0.18				−0.07				−0.08				0.02				0.09				**−0.32**	*	
31	E35M54-265	unm.			0.10				−0.10				−0.03				**0.28**	*			0.01				0.04		
32	E35M55-306	unm.			−0.10				0.18				−0.03				**−0.28**	**			−0.10				0.08		
33	E37M33-256	unm.			0.11				−0.03				0.07				**−0.31**	**			−0.10				0.13		
34	E38M50-135	unm.			−0.13				−0.03				0.00				0.17				0.03				**−0.28**	*	
35	E38M50-149	unm.			−0.09				**0.30**	*			0.18				−0.11				0.03				0.14		
36	E38M50-263	unm.			0.29	*			**−0.35**	**			−0.21				−0.09				−0.06				0.16		
37	E38M50-456	unm.			−0.02				**0.31**	*			**−0.35**	***			−0.04				−0.08				0.07		
38	E42M32-069	unm.			0.03				**−0.37**	**			0.15				0.10				−0.02				−0.17		
39	E42M32-156	unm.			−0.08				**0.37**	***			−0.06				−0.09				−0.04				0.02		

40	E42M32-160	unm.	−0.05		**0.35**	**	0.03	0.02		0.01	−0.03
41	E42M32-211	unm.	0.02		−0.16		0.07	**0.29**	**	0.00	−0.06
42	E42M32-228	unm.	−0.07		0.02		−0.11	0.04		−0.20	**0.30** **
43	E42M32-273	unm.	0.08		**−0.32**	*	0.07	0.06		−0.08	0.07
44	E45M49-176	unm.	0.03		0.00		−0.12	**0.39**	***	−0.09	−0.10
45	E45M49-226	unm.	0.12		**−0.32**	**	0.05	0.17		0.13	0.04
46	E45M49-388	unm.	−0.08		0.09		−0.15	0.05		−0.13	**0.28** *
47	E45M55-164	unm.	0.09		−0.12		0.10	0.12		−0.06	**0.27** *

[1] Marker map positions are based on an integrated map (Figure 2; Kraakman et al. 2004), except for the markers shown in italics, which were positioned based on the map of L94×116-5 (Qi et al. 2000) or L94×C123 (Qi et al. unpublished).
[2] Known QTLs: Reference to authors who reported a QTL in the same region. The authors were: (a) Kicherer et al. 2000, (b) Spaner et al. 1998, (c) Toojinda et al. 2000, (d) Scheurer et al. 2001, (e) Qi et al. 1998b, (f) Bezant et al. 1996, (g) Yin et al. 1999, (h) Chelkowski et al. 2003, (i) Jin and Steffenson 1994, (j) Hayes et al. 1993, (k) Backes et al. 2003, (l) Tinker et al. 1996, and (m) Marquez-Cedillo et al. 2001.
[3] The significance of correlation/regression is shown as *p*-value (simple testing), and as *q*-value (multiple testing). Significance of p *, **, ***, ****, $p < 0.01$, 0.001, 0.0001, 0.00001, respectively. Correlations printed in bold type had an FDR less than 5%.
[4] The RLP could only be measured on those cultivars that showed IT > 6.

Table 4. Occurrence of cultivars with a high or low Infection Type of *Puccinia hordei* isolate IVP2000 and allele for marker E39M61-255.

E39M61-255	Infection Type			
	Low (≤ 6)	High (> 6)	Unknown	Total
0	27	9	–	36
1	4	101	2	107
Unknown	2	3		5
Total	33	113	2	148

absence of the band (AFLP value zero). Table 4 shows that 27 of 36 cultivars with E39M61-255 = 0 had a low IT. Note that nine cultivars with E39M61-255 = 0 still had a high IT. All nine had the corresponding high AUDPC. Out of 107 cultivars with the susceptible phenotype for marker E39M61-255, 101 had a high IT, and four cultivars had low IT.

Another significant peak in association with IT was found on chromosome 2 around 138 cM. This peak coincides with a peak in RLP, and will be discussed later. Finally, one of the unmapped markers (E38M50-456) was highly associated with IT. In our set of cultivars this marker was moderately correlated with E39M61-255 ($r = -0.28$, $p = 0.001$), suggesting linkage to *Rph3*.

RLP, AUDPC: search for partial resistance for leaf rust

RLP and AUDPC are quantitative measures for leaf rust resistance. RLP is positively correlated with resistance level, since it is based on the time required for maturation of rust pustules in a monocyclic experiment. AUDPC is based on the level of infection in a field trial, and hence, is negatively correlated with resistance level. RLP was measured on cultivars with high IT, so without effective genes for hypersensitivity resistance. This way we concentrated on variation in level of partial resistance in the cultivar set, without confounding with hypersensitive resistance. For the same reason the LD analysis on AUDPC was carried out on those cultivars that had high IT in seedling stage.

AUDPC was significantly associated with four AFLP-markers on chromosome 7 between 69 and 82 cM. Presence or absence of E42M32-200 resulted in an average AUDPC of 216 or 229, respectively ($LSD_{0.05} = 7.2$). For E38M55-128 the

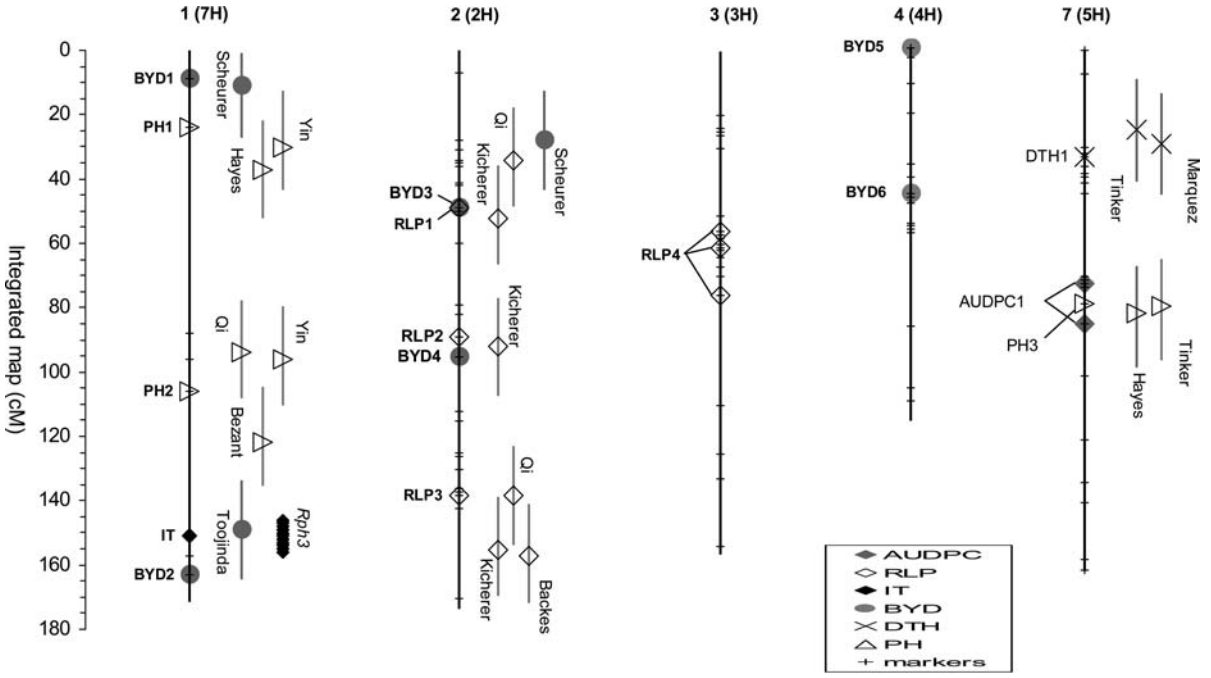

Figure 3. Overview of position of markers (symbols on chromosome bars) associated with traits (left of chromosome bars) and QTLs reported in literature (right of chromosome bars) for five barley chromosomes. The traits were leaf rust (*P. hordei*) resistance (IT = Infection Type, AUDPC = area under the disease progress curve for adult plants in the field, RLP = relative latency period of seedlings), BYD (Barley Yellow Dwarf), DTH (Days To Heading), and PH (Plant Height). The map distances are according to the integrated map (IM) as described by Kraakman et al. (2004). The positions of QTLs from literature have been recalculated to positions on the integrated map following: position IM = position literature×(chrom. length literature/chrom. length IM). All QTLs from literature have been drawn with 30 cM intervals. For the QTLs found with LD mapping only the associated markers were indicated, without intervals. Markers within 20 cM of each other were considered to be linked to the same QTL. The authors to which is referred can be found in Table 3.

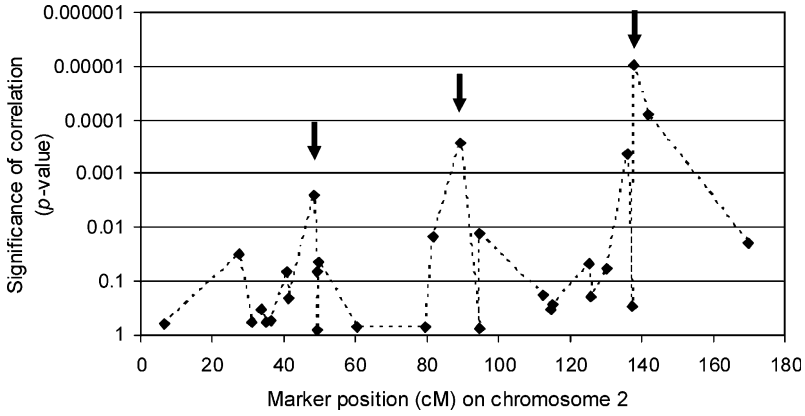

Figure 4. Association profile of RLP of *Puccinia hordei* on chromosome 2. The significance of correlation between marker and trait is shown. The position of three putative QTLs is indicated with an arrow.

AUDPC was 219 or 231, respectively ($LSD_{0.05}$ = 6.2). This locus had not been implicated before in mapping studies on field experiments in which partial resistance level was determined. Conversely, QTLs that had been reported to reduce AUDPC of *P. hordei*, like those

Table 5. Frequency and average RLP and AUDPC values of the most common marker genotypes in the *Rphq2* region. All other configurations occurred four or less times in the set of cultivars. For RLP the difference in subgroups is given based on Bonferoni corrected LSD(0.05).

E42M48-205 (Vada) 136.2 cM	E38M54-294 (L94) 137.4 cM	E38M55-251 (Vada) 137.7 cM	E42M48-376 (Vada) 141.8 cM	Count	Average RLP	Average AUDPC
1	0	1	1	19	112.5[a]	221
0	0	1	1	15	112.2[a,b]	224
0	1	0	0	33	109.6[b,c]	229
0	0	0	0	20	107.9[c]	229

of Vada reported by Qi et al. (1998b), did not appear in the present study.

RLP, measured in seedlings, was significantly associated with many markers (Table 3). If we consider markers within 20 cM of each other as indicative for one and the same putative QTL, we can distinguish three QTLs on chromosome 2 and one QTL on chromosome 3 (Figures 3 and 4). In addition, nine unmapped markers were associated with RLP. Out of the four QTLs indicated by LD mapping, three were at positions where QTLs for quantitative resistance to *P. hordei* had been reported before.

In Figure 4 an association profile is given for chromosome 2. Note that around 138 cM three markers had a very low *p*-value, so were associated with RLP, and one a high *p*-value. The markers with a significant *p*-value were all 'Vada-markers', i.e. the band for such a marker also occurred in Vada. The one marker that was not significantly associated with RLP (E38M54-294) was an 'L94-marker'. The marker that was most significantly associated with RLP was E38M55-251 ($r = 0.42$ $p = 9E\text{-}6$). The average RLP on the 40 cultivars with a band for that marker was 112.6, while the 62 cultivars without the band gave an average RLP of 108.9 ($LSD_{0.05} = 1.63$). Furthermore, when all possible marker configurations for the three Vada-markers and the one L94-marker were investigated, only four haplotypes occurred in the set of cultivars (Table 5). The most common configuration was the haplotype of L94. The 33 cultivars with this haplotype gave a low RLP. A comparably low RLP was found on the haplotype with only one marker difference (E38M54-294 changed from 1 to 0). Both haplotypes also gave a slightly (not significantly) higher AUDPC in the field. The Vada haplotype occurred 19 times, and these cultivars gave a significantly higher RLP than the ones with the L94 genotype, and a slightly lower AUDPC. A similarly high RLP was observed on the 15 cultivars with a genotype differing only for marker E42M48-205. Apparently, the allele change of this marker did not affect the level of partial resistance.

Table 6. Frequency of barley cultivars carrying *Rphq2* peak marker E38M55-251 with or without *Hordeum laevigatum* or Vada in their ancestry. Per subgroup the average RLP is shown. Tukey HSD (0.05) is given for the RLP averages per subset.

Ancestry contains	E38M55-251	Count	RLP	HSD
H. laevigatum				
0	0	11	107.7	a
1	0	42	109.3	a,b
0	1	3	111.5	a,b
1	1	34	112.8	b
Vada				
0	0	39	107.3	a
1	0	36	111.0	b
0	1	12	112.3	b
1	1	30	112.9	b

Marker E38M55-251 has been reported to be the peakmarker for an important QTL, *Rphq2*, for partial resistance against *P. hordei* (Qi et al. 1998b). This locus is at very short distance of powdery mildew gene *MlLa*, which has been reported to be contributed by *Hordeum laevigatum*, one of the parents of Vada (Arru et al. 2002; Jensen and Jørgensen 1991; Giese et al. 1993). Therefore, also *Rphq2* is likely to have been derived from *H. laevigatum*. In our set of cultivars, 113 cultivars had *H. laevigatum* in their ancestry, of which 70 also had Vada as ancestor. Analyzing only cultivars for which we both know their ancestry and their state for marker E38M55-251, 42 of the 76 cultivars derived from *H. laevigatum* and 36 out of 66 cultivars with

Vada in their ancestry did not carry E38M55-251 (Table 6). There was no association between the presence of *H. laevigatum* or Vada in the ancestry and the presence of E38M55-251 (χ^2 not significant). The average RLP on cultivars derived from *H. laevigatum* tended to be higher, but not significantly so, than on cultivars missing *H. laevigatum* in their ancestry (see HSD in Table 6). Vada in the ancestry was associated with a significantly higher RLP. However, when both the cultivar was derived from *H. laevigatum* or Vada and it carried marker E38M55-251 there was a significantly higher RLP.

Combined association for IT and RLP: chromosome 2 at 138 cM (Rphq2)

Three Vada-markers on chromosome 2 around 138 cM were positively correlated with RLP, and also positively correlated with IT (Table 3). This implies that they are associated to resistance (prolonged LP) and to susceptibility (high, compatible IT) at the same time. In our set of cultivars, only five out of 30 cultivars (17%) with low IT had marker E38M55-251, the peak marker for *Rphq2*, while 40 out of 104 cultivars (38%) with high IT had this marker. The difference is highly significant (χ^2; p = 1.5E-5). This suggests that if in breeding programs advanced germplasm carried an effective gene for hypersensitivity (low IT) breeders relatively frequently did not select *Rphq2*, since they could not judge the level of partial resistance.

Linkage disequilibrium mapping of other traits

BYD: many associated markers

Many markers were found to be associated with the level of BYD. Assuming that markers within 20 cM of each other indicate the same putative QTL, we found two QTLs each on chromosome 1, 2, and 4 (Table 3, Figure 3). Furthermore, six unmapped markers were significantly associated with BYD symptoms (Table 3).

Special notice should be taken of SSR marker HVM054 on chromosome 2. With an r of 0.67 (p = 9E-15) the association between this marker and BYD was extremely significant. Table 7 shows that HVM054 had five different alleles in the cultivars, of which one resulted in a significantly lower score for BYD. The mean BYD value for SSR allele size 158 bp was 0.993, indicating that cultivars carrying this SSR allele were much more resistant or tolerant to BYD than the cultivars carrying any of the other four alleles (mean symptom level at least 2.287). An analysis of the ancestries of all 15 cultivars did not reveal any common ancestor which could have donated the resistance gene.

Three of the six QTLs for BYD resistance indicated by LD mapping have been reported earlier (Table 3, Figure 3). QTLs on chromosome 1 and 2 coincided with QTLs reported by Toojinda et al. (2000) and Scheurer et al. (2001). The other three QTLs have not been reported before, including the QTL that was highly associated with one allele of HVM054 (Table 7). There was high correlation, but just below the level taken as threshold for significance, for markers on chromosome 3 at 154 cM (r = 0.24, p = 0.0057), on chromosome 4 at 86 cM (r = −0.25, p = 0.0036), and on chromosome 5 at 98 cM (r = −0.24, p = 0.0039). In all those chromosome regions Toojinda et al. (2000) found QTLs for BYD. In addition, BYD score correlated significantly with rachilla hair length (Table 2), which mapped on chromosome 7 (see below). This indicates an additional, previously unreported, BYD resistance gene on chromosome 7.

Table 7. Mean BYD scores of five subsets of barley cultivars according to HVM054 allele. The different subsets are based on Tukey HSD(0.05).

HVM054 (allele migration on gel in bp)	N	Average BYD score
150	45	2.682
154	11	2.287
158	15	0.993
162	5	2.399
167	57	2.679

DTH and PH

For the trait DTH one QTL was found on chromosome 7 at 32 cM, and one unmapped marker was significantly associated. For PH, associated markers were found on chromosome 1 and 7. Besides, eight unmapped markers were significantly associated with PH. In total, three QTLs for PH were indicated with LD mapping. On the positions of all these three indicated QTLs genes for PH had been reported before (Table 3, Figure 3).

Rachilla and lodicule size

Rachilla hair length was associated ($r = 0.36$; $p = 0.0027$) with marker Bmag223 on chromosome 7 at 76.2 cM, a marker known to be closely linked to the gene *srh* for rachilla hair length (Costa et al. 2001). Lodicule size associated markers were found on chromosome 4 around 50 cM.

Discussion

Linkage disequilibrium existed in this set of modern spring barley cultivars. This LD could be used to associate a variety of traits with a set of 236 AFLP marker loci and 11 SSR marker loci. We found major genes as well as minor genes, for morphological, resistance, and for other agronomically relevant traits. Many of the trait-associated markers were located in a region where other authors had reported QTLs for the same trait after linkage studies with segregating populations. Especially for leaf rust resistance and BYD, the LD mapping suggested interesting novel QTLs.

The integrated map contained 128 AFLP markers and 11 SSR markers (Figure 2). Assuming that genetic positions up to a distance of 10 cM are in LD (Kraakman et al. 2004), each marker on the map would in principle allow detection of LD with a trait locus up to 10 cM in either direction along the linkage group. Therefore, if two adjacent markers on the integrated map border a gap of 20 cM, they would still cover the interval for LD detection, but if the gap between the markers exceeds about 20 cM, the stretch beyond 10 cM from the flanking markers can be considered uncovered. The integrated map contained 14 of such intervals larger than 20 cM, resulting in a total of at least 208 cM (19.8% of the genetic map) where detection of LD was unlikely, because of paucity of markers. Especially on chromosome 1 there are large gaps. Future mapping of the trait-associated markers (markers 24–47 in Table 3) may fill some of the gaps and allow positioning of additional genes for the traits considered here.

Rust resistance

In Europe, *Rph3*, is one of the most frequently used genes for hypersensitivity resistance against *P. hordei* in modern cultivars, although virulence against this gene occurs at moderate frequencies (Niks et al. 2000; Dreiseitl and Steffenson 2000). It is therefore not surprising that the IT scored on the set of cultivars correlated very well with markers in the region of *Rph3* on chromosome 1. The absence of a band for AFLP marker E39M61-255 was associated with resistance. Apparently, the marker band occurred in European spring barley germplasm at a frequency of nearly 1 before the introduction of *Rph3*, but the donor possessed, linked to *Rph3*, the rare 0 allele of the marker. The occurrence of nine cultivars (Table 4), which lacked the band but did have high IT, could be due to a number of reasons. For instance, these accessions may have obtained a different 0 allele of the marker from another, non *Rph3* carrying source. Also, linkage could have been broken between this marker and *Rph3*.

Four cultivars, Hanka, Abed5193, Enigma, and Tofta, had the marker of E39M61-255 that is associated with the susceptibility allele *rph3*, but they showed hypersensitivity (IT 3 or 4 on the 0–9 scale). Their resistance is probably due to effective *Rph* genes elsewhere on the genome. Remarkable in this group was Hanka, probably carrier of *Rph7* (Niks et al. 2000). In seedlings, the resistance was effective (IT = 3), but in the field, Hanka showed a surprisingly high AUDPC (215). In previous trials, Hanka had high resistance also in the field, and virulence to *Rph7* still has not been reported in the European *P. hordei* population (Niks et al. 2000). We have no explanation for this unexpected result on Hanka.

QTLs for rust resistance have been reported by many authors, e.g. Qi et al. (1998b, 1999, 2000), Kicherer et al. (2000), and Spaner et al. (1998). Qi et al. (1998b, 1999) consistently found QTLs for RLP: *Rphq2*, *3*, and *4*. The LP prolonging alleles were contributed by Vada, a cultivar that features in the ancestry of 70 of the 148 cultivars. LD mapping in the cultivars showed that *Rphq2* on the distal part of chromosome 2 was also present and effective in this germplasm. However, in the regions of *Rphq3* and *Rphq4*, on chromosomes 6 and 7, no markers were found to be associated with RLP or AUDPC. First, *Rphq2* will be discussed, and then *Rphq3* and *4* will be elaborated on.

Rphq2:

Rphq2 was found in our set of cultivars with significant association between RLP and three AFLP markers on chromosome 2. The same markers were also found by Qi et al. (1998b) to be peak markers for *Rphq2*. In contrast to Qi et al., these markers were not associated with AUDPC. One marker within the *Rphq2* region (Qi et al. 1998b) was not associated with RLP: E38M54-294 (Figure 4). The allele giving an amplification product was from L94 (L94-marker), and would be expected to give a strong negative association with RLP. In the L94×Vada RIL population of Qi et al. (1998b), E38M54-294 was associated with RLP. In the RIL population, there is no doubt that the band on gel is from L94, but in the cultivar set the band could also originate from another part of the genome and co-migrate by identical mobility (Waugh et al. 1997; Koopman and Gort 2004). However, the association between E38M54–294 and the Vada markers was just as high as the mutual correlation between the Vada markers in the *Rphq2* region, indicating that no other band has been interfering. The lack of association in our germplasm might be due to the fact that breeding germplasm lines that carry the L94-allele, without carrying an effective *Rph* gene, would tend to have an unacceptably low level of partial resistance. The breeder would reject them, unless the absence of *Rphq2* is compensated by the presence of (an)other QTL(s) elsewhere on the genome. This would lead to the interesting hypothesis that, for characters for which many loci can contribute, association between marker alleles and positive, agronomically desirable alleles, is more likely to be found than association between marker alleles and agronomically undesirable alleles.

Rphq3 and 4

In this set of cultivars, we did not find association between markers and RLP or AUDPC that could indicate variation for *Rphq3* on chromosome 6 and *Rphq4* on chromosome 7. Among the possible explanations for this lack of association between *Rphq3*- and *Rphq4*-linked markers and RLP and AUDPC are the following: (1) Maybe the indicative markers are not sufficiently close to the loci for partial resistance to have resulted in LD, (2) maybe loci for other agronomically relevant characters are linked to the locus for partial resistance, but with the favorable alleles in repulsion phase, stimulating recombination and/or selection against the allele for partial resistance, or (3) the partial resistance-enhancing effect of *Rphq3* and *Rphq4* may depend on interaction with other genes in the genotype, making their effect genotype dependent. Support for the latter explanation can be found in NILs that have been developed from the L94×Vada population (Berloo et al. 2001). It appeared that *Rphq4* was only moderately effective in a L94 background (pers.comm. Niks and Marcel).

AUDPC was only associated with markers on chromosome 7 around 70 cM. Those markers were also present and segregating in the L94×Vada population, but did not show association with AUDPC there. This is unexpected, and further research is required to explain this apparent contradiction. It was the only association with AUDPC found in the present set of cultivars.

BYD

BYD was associated with many mapped and unmapped markers, indicating putative QTLs on chromosome 1, 2, 4 and 7. The indicated chromosome regions seem to coincide with one QTL that had been reported by Toojinda et al. (2000) and two QTLs reported by Scheurer et al. (2001). Remarkably, the commonly used major gene *Ryd2* for BYD resistance, located on the long arm of chromosome 3 (Collins et al. 1996), was not found with LD mapping, although it cannot be ruled out that one or more of the unmapped markers is linked to *Ryd2*. Another possibility is that in the set of cultivars *Ryd2* is not present. Testing the material for the *Ryd2* linked markers YLM (Jefferies et al. 2003) and YLP (Ford et al. 1998) would indicate which and how many cultivars are likely to possess *Ryd2*.

New putative QTLs for BYD have been found on chromosomes 1, 2, 4 and 7. Of special interest is SSR marker HVM054 on chromosome 2. The association between this marker and BYD was extremely significant. One SSR allele is associated with a very low symptom score compared to the other four SSR alleles. To the best of our knowledge, no gene for BYD resistance or tolerance has been reported in this region. Fifteen cultivars carried the marker allele associated with

the resistance. They had no obvious common ancestry.

DTH and PH

DTH was significantly associated with only two markers. This may seem surprising, as many QTLs for heading date have been reported. In a mapping population of Blenheim × Kym, Bezant et al. (1996) found QTLs for DTH on each chromosome, and on some chromosomes even more than one. This showed that QTLs for DTH are abundantly present even in one biparental mapping population, so we may assume that in a set of cultivars with many different ancestors a large number of QTLs affecting DTH may be present and effective. It will then be hard to detect LD between a marker and one of the many QTLs. Furthermore, the range of DTH values for all cultivars was rather small (Table 1), suggesting high adaptation for earliness of all genotypes. Another possible explanation for finding only two QTLs for DTH may be that breeders all have predominantly relied on the same QTLs for DTH, leading to lack of genetic variation in QTLs and associated markers in the set of cultivars.

PH was associated with three mapped markers and with eight unmapped markers. All mapped associated markers were in a region where QTLs for PH have been reported before. Similar to DTH, many QTLs for PH have been reported. In contrast to DTH, for PH we did find four QTLs with LD mapping. This suggests that for PH fewer QTLs are present in the cultivar germplasm than for DTH, so LD between a marker and a QTL was less obscured by the possible compensation at other QTLs.

Combined association for IT and RLP: chromosome 2 at 138 cM (Rphq2)

Cultivars carrying *Rph3* were less likely to carry *Rphq2* for partial resistance to *P. hordei*. We presume that if in breeding programmes advanced germplasm carried an effective gene for hypersensitivity (low IT), breeders relatively frequently did not select *Rphq2*, since they could not judge for level of partial resistance. This may be regarded as evidence supporting the theory of the 'Vertifolia effect'. This effect has been defined by Vander-Plank (1963) as the loss of horizontal resistance in the process of breeding for vertical resistance. Parlevliet (1981) argued that, however logical the 'Vertifolia effect' appears, it is not a general phenomenon. As example of evidence against this effect, he mentioned the barley cultivar 'Cebada Capa' which combines a widely effective gene for hypersensitive complete resistance to *P. hordei* (*Rph7*), but has a high level of partial resistance in its genetic background (Parlevliet and Kuiper 1977). This evidence is based on an example of a possibly casual cultivar, whereas the evidence appearing from our study is based on frequencies in a wide set of cultivars.

Rachilla and lodicule size

The marker associated with rachilla hair length was associated with the SSR marker Bmag223 on chromosome 7. This marker mapped less than 3 cM from the gene *srh* for rachilla hair length in several barley mapping populations (L94 × Vada and Steptoe × Morex by T.C. Marcel, unpublished data; Oregon Wolfe Barley Costa et al. 2001). This was another example where LD mapping is in agreement with mapping data from biparental mapping populations.

Lodicule size was associated with markers on chromosome 4 around 50 cM, rather than on chromosome 2 around 150 cM, as reported for this trait by Turuspekov et al. (2004). Interestingly, both areas are known to harbour several loci for *Fusarium* Head Blight resistance, a trait that tends to be associated with closed flowering (Zhu et al. 1999; De la Peña et al. 1999). Since large lodicules play a role in open flowering habit, exposing the interior of the florets to *Fusarium* spores, our data suggest that the locus on chromosome 4 indeed contributes to lodicule size.

The value of LD studies

A number of the associations between markers and trait values were for markers located in a region where already QTLs for the trait considered had been reported. This indicates that QTLs detected in mapping populations from biparental

mapping populations were widely represented in this set of cultivars, and that they could be detected with LD mapping. Associations between traits and markers in regions that had not been implicated before to affect the trait suggest new QTLs. Such new QTLs were found for AUDPC, RLP, and BYD. Probably, those traits have never been studied in a mapping population where both parents differed for the QTLs involved, or there were no markers in that region. The newly suggested QTLs in the present study should be validated in a study with an appropriate mapping population. Such a population should segregate for the contrasting alleles of the associated marker. Our paper indicates that LD studies are efficient in indicating novel genes for important agronomic characters that subsequently can be validated in specific biparental crossing populations, and in confirming QTLs that have been detected in biparental mapping populations.

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