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QTL mapping for resistance against non-parasitic leaf spots in a spring barley doubled haploid population

Abstract Phenotypic variability for resistance against non-parasitic leaf spots (NPLS) has been observed between varieties. For the genetic characterization of NPLS resistance, a population with 430 doubled haploid (DH) lines was developed from the cross between the NPLS-resistant *Hordeum vulgare* breeding line IPZ24727 and the NPLS-sensitive barley cultivar Barke. A molecular map was constructed based on 164 AFLPs, 30 SSRs and one STS marker derived from the *mlo* gene. Field trials were performed over four environments in which NPLS and other agronomic traits were assessed. Estimates of genotypic variance were highly significant for NPLS. Moreover, no transgression was found for the trait. Quantitative trait loci (QTLs) for NPLS resistance were mapped in the DH population on chromosomes 1H, 4H, and 7H, with the most important effect on chromosome 4H. The QTLs for NPLS explained together 39% of the phenotypic and 49% of the genotypic variance, thereby showing additive gene action. Consequently, marker-assisted selection for improving NPLS resistance is possible.

Introduction

Non-parasitic leaf spots (NPLS) are a relatively recent type of plant damage, appearing in cereals but primarily in barley. These spots were observed on barley plants in

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southern Bavaria more than 10 years ago and have also been visible in other German regions and European countries. NPLS can result in yield losses of up to 22% in spring barley and up to 40% in winter barley trials (Baumer et al. 2001). Despite the growing economic importance of NPLS, the main cause remains unknown. To date, no pathogen or bacteria, neither virus nor animal and no inappropriate pesticide applications have been determined to be responsible for NPLS (Obst and Huber 1996). It is known that NPLS appear just after the heading date and are enhanced under conditions of high solar radiation following wet weather.

Non-parasitic leaf spots should be distinguished from other physiological leaf spots. The latter category can include cultivar-specific spots or spots caused by nutrient deficiency or by the *mlo* resistance gene (Obst and Gehring 2002) and are phenotypically distinguishable from NPLS. NPLS are small, often round spots that appear just after heading date and begin in the areas of the leaves that are directly exposed to solar radiation. On leaves that are in the shadow, overlapped or turned, no or only small spots have been observed. The damage depends on the intensity of the solar radiation and the developmental stage of the plants and leaves. NPLS start on older leaves (F-2) and then continue up to the flag leaf. Later, they also appear on the leaf sheath, the culm, the ear and the awn (Baumer et al. 2001).

A high number of NPLS reduces the photosynthetic rate, resulting in a premature end in the accumulation of reserve material. Within only a few days the number of spots increases, and in the end the leaves are completely necrotic. In addition, plants become more susceptible to secondary parasites. Affected plants ripen nearly 3 weeks earlier, resulting in a reduced yield, kernel size and malt quality (Baumer et al. 2001).

To date, barley has been observed to be the most sensitive crop to non-parasitic leaf spots. Two extreme genotypes with respect to NPLS sensitivity were utilized as parents for the doubled haploid (DH) population used in the investigation reported here. As NPLS are of increasing economic importance, the aims of the project

were (1) the localization and characterization of quantitative trait loci (QTLs) for NPLS resistance in spring barley; (2) the determination of possible correlations between NPLS resistance and other agronomic traits; (3) the evaluation of the usefulness of a marker-assisted selection (MAS) for NPLS.

Materials and methods

Plant material

An anther culture-derived DH population of 430 DH lines was developed from F_1 hybrids of the cross of the NPLS-resistant spring barley (*Hordeum vulgare* L.) accession IPZ24727 and cv. Barke. The parent IPZ24727 derives from an Israeli wild species and possesses a good resistance against NPLS.

Field experiments

Field trials were performed over 3 years in two replications of two-rowed plots. The DH lines were assessed in two locations in southern Bavaria [in Frankendorf for 3 years (2000–2002) and in Irlbach in 2002].

The degree of NPLS severity was scored on the upper three leaves (F-2, F-1 and the flag) as the percentage of damaged leaf area. Subsequently, heading date, plant height, powdery mildew infestation, *mlo* spots, 1,000-kernel weight (TKW) and kernel size were assessed. The NPLS symptoms appeared within a short period of time after heading, so the scoring had to be done within approximately 2 weeks. Pesticide applications excluded the use of fungicides.

Pathogen diagnostics

For NPLS scoring, leaves were removed from the parents and eight DH lines that differed in NPLS susceptibility. The leaves were placed in petri dishes according to Sachs et al. (2000) and examined after 3 days under a microscope at a 30-fold magnification. The objective was to detect and so to avoid the assessment of spots caused by the pathogen *Ramularia collo-cygni*, Sutton and Waller.

DNA isolation

Genomic DNA was isolated according to Anderson et al. (1992) with some modifications. Eight barley leaf discs 7 mm in diameter were lyophilized and powdered by ball mill in 96-well plates. Extraction buffer with 3.9 g/l sodium bisulphite was then added to the plant material and the mixture was incubated at 65°C for 30 min. A 20-ml aliquot of chloroform/isolamylalcohol (24:1) was added twice, where the aqueous phase was at both times transferred into new tubes after centrifugation (10 min, 5,000 rpm). The RNA was then removed with 1 μg RNAse. DNA was precipitated by adding isopropanol for 30 min, followed by centrifugation at 5,000 rpm for 3 min. The supernatant was discarded, and the samples were washed first with sodium acetate (0.2 *M*) in 76% ethanol for 30 min and second with NH_4-acetate (10 m*M*) in 76% alcohol. The DNA remaining in the well-plates was dried under vacuum and dissolved in 50 μl of 1× TE-buffer (pH 8.0), reaching a concentration of about 50 ng/μl.

Amplified fragment length polymorphism analysis

The AFLP analyses were carried out according to Vos et al. (1995) with some modifications (Hartl et al. 1999). Genomic DNA (250 ng) was digested with the restriction enzymes *Pst*I (2.5 U) and *Mse*I (1 U) for 2 h. The *Pst*I-adapter (2.5 pmol) and *Mse*I-adapter (25 pmol) were added to the mixture to ligate them to DNA fragments using 1 U T4-DNA ligase for 8 h. The selective amplification was carried out in 10 μl reaction volumes with *Pst*I/*Mse*I primer combinations, each with three selective bases. All amplifications were carried out in PTC-200 MJ-Research (Waltham, Mass.) and 9600 Perkin Elmer (Foster City, Calif.) thermocyclers.

Simple sequence repeat analysis

For the Bmag, Bmac, EBmac (Ramsay et al. 2000) and GBM (Thiel et al. 2003) microsatellites, 20 μl reaction volumes were used (60 ng DNA, 1× PCR buffer, 0.2 m*M* dNTPs each, 2.5 m*M* $MgCl_2$, 0.5 μ*M* SSR primer and 0.25 U *Taq* polymerase). The GMS (Struss and Plieske 1998), the HVM (Becker and Heun 1995; Liu et al. 1996) and the MGB (Pillen et al. 2000) microsatellites were analysed in 10 μl reaction volumes under the same conditions as the 20 μl reaction volumes. The PCR programmes consisted of an initial denaturing step at 94°C for 2 min, followed by the annealing step (40 cycles of denaturation at 94°C for 30 s and extension at 72°C for 1 min). The annealing temperature was decreased in 1°C increments from a temperature 10°C higher than the optimal temperature in the first cycle until it reached the optimal temperature, where it was kept constant for the remaining cycles. The optimal annealing temperature varied depending on the SSR primer. The programmes ended with a primer extension step for 5 min.

DNA fragments were separated through 5% denaturing polyacrylamide gels under standard conditions and scanned in a Typhoon 9200 (Amersham, Braunschweig). Unlabelled microsatellites were stained with Vistra Green (Amersham, Braunschweig).

Molecular map

The map was constructed with MAPMAKER (Lander et al. 1987), using a logarithm on odds (LOD) threshold of 4.0 and 25 cM as the maximal distance between two linked markers. A total of 86 DH lines were used to generate a primary chromosomal map. Microsatellites were used to assign linkage groups to the different barley chromosomes and to discard possible heterozygous lines within the population. The molecular map was ultimately constructed with all of the DH lines based on 164 polymorphic AFLP markers, 30 microsatellites and the sequence-tagged site (STS) marker from the *mlo* gene. The latter was assessed by EpiGene (Freising).

Linkage groups were validated and linked if feasible using JOINMAP 3.0 (van Ooijen and Voorrips 2001). The Haldane mapping function was assumed when using MAPMAKER and JOINMAP (Haldane 1919). Some of the DH lines were discarded, with 430 DH lines ultimately being used. AFLP markers with a deviation from Mendelian segregation (*P*<0.001) were eliminated for map construction.

QTL analysis

Phenotypic and molecular marker data were integrated for QTL analysis performed by PLABQTL VER. 1.1 (Utz and Melchinger 2000). The phenotypic data consisted of the means of field scores for NPLS and agronomic traits over all environments. The number and position of QTLs, their additive effects and the explained phenotypic and genotypic variances were calculated. Final QTL analysis was carried out by composite interval mapping using the nearest marker of each QTL as a cofactor for the trait in study. Digenic epistatic effects between QTLs were detected by the "smodel function". The F-to-enter value was set to 15. The LOD score utilized for QTL detection was above 3.0.

Table 1 Parental means and statistical data from the DH population expressed as a mean over environments (2000–2002)

Trait		Parents[a]		DH population[b]							
Code[c]	Units	μ_{IPZ}	μ_B	μ_{DH}	Median	Mode	Minimum	Maximum	variance	Standard deviation
NPLS	Percentage leaf damage	2.7	21.3	15.3	15.0	9.5	4.0	29.4	32.1	5.7
Mt	Units	1.2	1.0	1.8	1.2	1.0	1.0	5.7	1.3	1.1
PH	Centimeters	79.2	72.3	75.2	75.4	73.8	66.8	84.0	9.3	3.1
HD	Days after 1 June	17.0	17.0	17.2	17.3	17.1	13.5	20.1	1.6	1.3
TKW	Grams per 1,000 kernels	47.4	47.5	47.3	47.3	48.4	40.0	54.1	5.1	2.3
>2.5 mm	Percentage	81.6	73.8	76.1	77.1	74.1	38.4	92.5	54.8	7.4

[a] μ_{IPZ}, Means of IPZ24727; μ_B, means of Barke
[b] μ_{DH}, Means of the DH population; minimum, lowest score; maximum, highest score
[c] NPLS, Non-parasitic leaf spots scored on the three upper leaves; Mt, powdery mildew infestation; PH, plant height; HD, heading date; TKW, 1,000-kernel weight; fraction with a kernel size >2.5 mm

Statistical analysis

Statistical analyses were carried out with SAS version 8.0 (SAS Institute 2000). Normal distribution of trait scores was tested with the Shapiro-Wilk-test by the PROC UNIVARIATE procedure. The correlations between scored traits or environments were calculated with the PROC CORR procedure. Analyses of variance were performed with GLM (general linear models). Not normally distributed data were tested with NPAR1WAY. The calculation of heritability in the broad sense was performed by PLABQTL (Utz and Melchinger 2000). Distorted segregation of markers was determined with the χ^2-test. The similarities of DH lines were controlled with JOINMAP 3.0 where a DH line that has more then 90% genetic similarity with another DH line was dropped, thereby obtaining a non-redundant dataset and more secure map distances.

Results

Phenotypic data of NPLS and agronomic traits

NPLS scoring was carried out when the symptoms first appeared, thereby avoiding the assessment of damage caused by other pathogens or possible secondary parasites. No fungus (*Ramularia collo-cygni*) was found during NPLS scoring, although it did appear later in the season on some leaves, as did the saprophytic pathogens *Botrytis cinerea*, *Cephalosporium sp.*, *Cladosporium sp.* and *Penicillium sp.* (P. Büttner, personal communication).

The mean of the resistant parent, IPZ24727, and that of the susceptible parent, Barke, greatly differed with respect to NPLS (Table 1). Within the DH population a high phenotypic variability for NPLS was also observed. Otherwise, no large differences either between parents or within the population were observed for the traits powdery mildew infestation, plant height, heading date and TKW. The overall NPLS mean from the population differed between environments, varying from 6% leaf damage caused by NPLS in 2000 to 15% in 2002. Frankendorf 2002 showed the strongest scored symptoms. Nevertheless, highly significant correlations of about 0.6 were found between environments (P<0.01). In addition, no transgressions were observed for NPLS.

The distribution of the phenotypic means of NPLS (Fig. 1) followed an approximately normal distribution. In
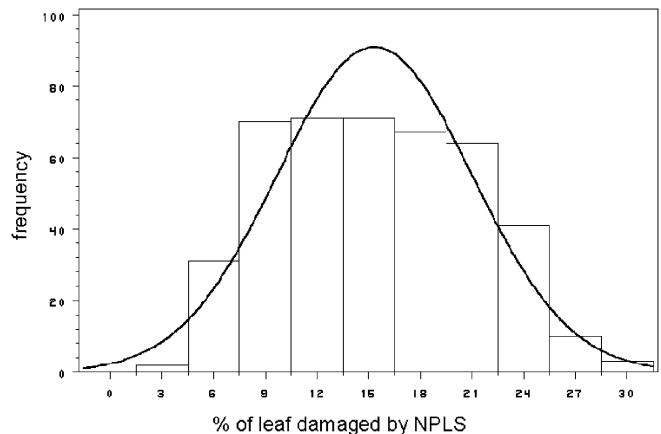
Fig. 1 Histogram for NPLS means over all environments

Table 2 Analysis of variance for NPLS across four environments

Item	df	MS	F value	P value
Environment (E)	3	190,338.7	11,188.6	<0.0001
Genotype (G)	429	254.5	14.96	<0.0001
G×E	1,287	49.8	2.93	<0.0001
Error	1,702	28,954.0		

contrast, the assessment of powdery mildew infestation showed a positive skew with a high proportion of powdery mildew-resistant DH lines. This skew was caused by the presence of the *mlo*-mildew resistance gene inherited by Barke that conferred a complete resistance against powdery mildew.

Genotypic (G) variances as well as the environment (E) variances and the G×E interactions were highly significant for NPLS (Table 2). Heritability for the scored traits was moderate to high (Table 3), while heritability for NPLS was 80%. The correlations among NPLS and the other scored traits were highly significant but of rather low to moderate values. NPLS and powdery mildew infestation showed the highest correlation of −0.45 (Table 4).

Table 3 Heritability of scored traits

Parameter[a]	Traits[b]					
	NPLS[(4E)]	Mt[(3E)]	PH[(4E)]	HD[(4E)]	TKW[(3E)]	>2.5 mm[(3E)]
H^2	0.80	0.86	0.76	0.76	0.60	0.73
95% C.I.	0.77–0.83	0.83–0.88	0.72–0.79	0.71–0.79	0.53–0.66	0.68–0.77

[a] H^2, Heritability; 95% C.I., confidence interval with $\alpha=5\%$
[b] NPLS, Mt, PH, HD, TKW, >2.5 mm, see Table 1 for codes; (3E), (4E), heritability based on three or four environments, respectively

Table 4 Genotypic and phenotypic correlation matrix from the means of the DH population (2000–2002)

Trait[a]	NPLS[(4E)]	Mt[(3E)]	PH[(4E)]	HD[(4E)]	TKW[(3E)]	>2.5 mm[(3E))]
NPLS		−0.45**	−0.27**	−0.20**	−0.22**	−0.24**
Mt	−0.59++[b]		0.00	0.00	0.11*	0.18**
PH	−0.40++	−0.01		−0.12*	0.44**	0.30**
HD	−0.23++	0.00	−0.14++		−0.08	−0.20**
TKW	−0.41++	0.13+	0.62++	−0.10+		0.61**
>2.5 mm	−0.36++	0.24++	0.36++	−0.26++	0.77++	

*, ** Phenotypic correlation was significant at the 0.05 and 0.01 probability levels, respectively
[a] See Table 1 for abbreviations of the trait codes; (3E), (4E), calculations based on three or four environments, respectively
[b] +, ++, Genetic correlation exceeded one or two times its standard error, respectively (Utz 2001)

Table 5 QTLs for NPLS based on 430 doubled haploid lines

QTL	Chromosome	Position	Left marker	LOD	R^{2a}	Additive effect
QNpls.lfl-1H	1H	75	P72M50-349	4.1	4.3	1.14
QNpls.lfl-4H	4H	65	EBmac0635	38.6	34.3	4.28
QNpls.lfl-7H	7HS	25	P68M52-163	3.0	3.4	0.53
Adjacent R^2					39.0	

[a] R^2, Proportion of phenotypic variance explained by the respective QTL

Map construction

A preliminary linkage map was constructed with 86 DH lines. A set of 14 AFLP primer combinations that covered the mapped genomic regions well was then used to analyse all 430 DH lines, thereby extending the map to all individuals of the population. The molecular map was finally constructed on the basis of 164 polymorphic AFLPs, 30 microsatellites and the *mlo* marker. Chromosomes 2H, 3H, 4H, 6H and 7H comprised two or three linkage groups. The resulting map length was about 1,092 cM with an average interval length of 2.3 cM (Fig. 2).

QTL analysis for NPLS

For NPLS, three QTLs were detected on the DH population over all environments with a LOD>3.0. These explained together 39% of the phenotypic and 49% of the genotypic variance. The most noticeable QTL was mapped near the *mlo* locus on chromosome 4H, and it explained 34% of the phenotypic variance and appeared in each environment. The other QTLs for NPLS mapped on chromosomes 1H and 7HS, explaining 4.3% and 3.4% of the phenotypic variance, respectively. QTLs for NPLS showed no digenic epistatic effects. The alleles increasing

the resistance were all contributed by the resistant parent, IPZ 24727 (Table 5).

Some QTLs for NPLS mapped together with QTLs for other traits. The QTL QNpls.lfl-1H was localized together with a QTL for heading date, although only low genetic correlations were found between both traits. Further on, QNpls.lfl-4H was mapped together with a powdery mildew resistance QTL, where both QTLs showed large effects on the expression of resistance for the respective trait. The QNpls.lfl-7H clustered with QTLs for powdery mildew resistance, plant height and TKW.

Discussion

Phenotypic and genotypic characteristics of NPLS

The population of 430 DH lines derived from the cross IPZ24727/Barke proved to be adequate to study the resistance against NPLS. The large difference in NPLS severity of the parents as well as the high phenotypic variability and heritability (80%) of NPLS within the DH population provided suitable information about the genetic determination of NPLS resistance. Our results confirmed that the severity of NPLS is greatly influenced by environmental factors, showing big differences in the population mean between scored environments, although the correlation coefficients between them stayed high.

Fig. 2 Linkage map of the DH population IPZ24727/Barke. QTLs are displayed with the corresponding trait abbreviation. See Table 1 for explanation of codes
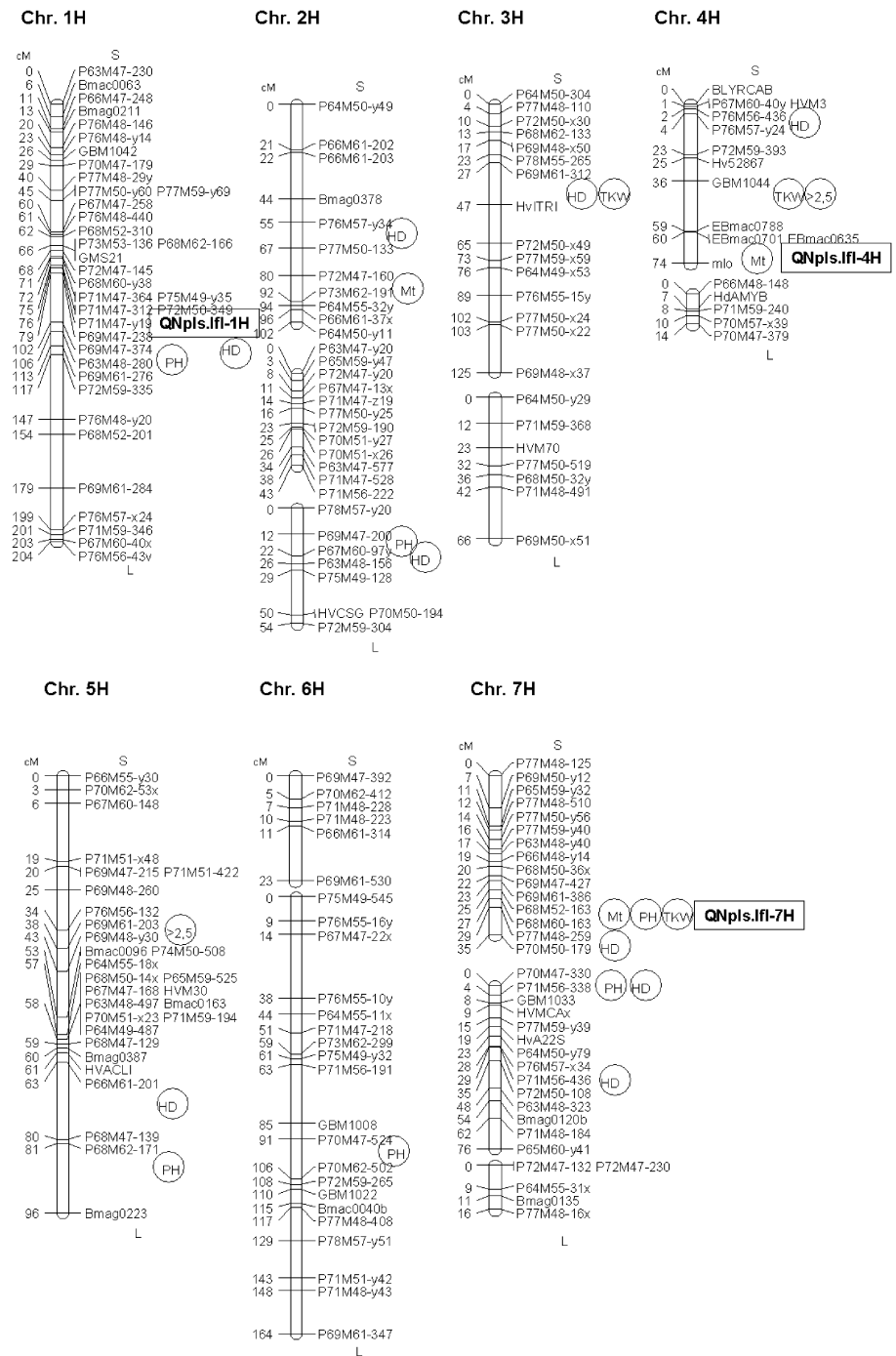
Even correlations between different scored leaves, such as F-2, F-1 and the flag, showed high significant coefficients (approximately 0.90) (data not shown).

As NPLS are a rather new damage phenomenon, their cause and resistance mechanism remain unknown. One proposal has been that NPLS symptoms in barley appear as a reaction towards an enhanced oxidative stress: barley leaves that have reached their final size are not able to compensate for the high amounts of reactive oxygen species (ROS) caused by sudden changes from wet weather conditions to high solar radiation, resulting in the production of NPLS. It is known that the damage is not caused by pathogens, as revealed by several researchers (Obst and Huber 1996, Baumer et al. 2001, J.-P. Schnitzler, personal communication) and the observations made during the present study. Although no relationship has been found between NPLS and plant diseases like *Rynchosporium secalis* or *Puccinia hordei*, genotypes carrying the *mlo* powdery mildew resistance gene showed an increased susceptibility to NPLS. Moreover, NPLS have been induced in the greenhouse with metal halide lamps and constant light conditions from EC 32 on, where

the genotypic variability of those barley plants was similar to that in the field, but with less severe symptoms (data not shown).

Breeding line IPZ24727 shows the best NPLS resistance of all the cultivars, and *H. spontaneum* wild barley also has a good NPLS resistance (data not shown). As wild barley would be a useful source of abiotic stress tolerances, it has been supposed that the resistance of IPZ24727 derives from the Israeli wild barley crossed during the development of the breeding line. This breeding line was a parent of the DH lines used in the present study.

Molecular mapping and QTL detection

The distorted segregation of markers is a common phenomenon in DH lines developed in anther culture (Graner et al. 1991). Although markers with a deviation from the Mendelian segregation were eliminated, segments with distorted segregation were found on chromosomes 5H and 7HL in the DH population. The biased segregation found on chromosome 7H was towards the parent Barke. Sayed et al. (2002) explained that the distorted segregation on 7H was mainly due to genetic effects, where the preferential regeneration of gametes comes from the parent that responds better to tissue culture.

The low rate of polymorphism between parents was principally evident from the few polymorphic SSRs within the population. The resistant parent IPZ24727 derives from a wild barley but has been crossed several times with German cultivars like the cultivar Trumpf, which is also an ancestor of the NPLS-susceptible parent Barke. Sections of the genome that are not polymorphic should not contain segregating genes. So, despite the fact that some genomic regions could not be mapped, marker intervals of linkage groups were close enough, reaching a good coverage of the barley genome and thus aiding QTL detection.

QTL detection power could be increased by using large populations and population types like double haploids (Asins 2002) as well as by high heritabilities and by choosing extreme genotypes in the trait chosen as parents for the population (Lander and Botstein 1989). The population size of the double haploids in this study and the relative high heritability of NPLS thus improved QTL detection. In addition, the parents showed extreme genotypes with respect to NPLS but few differences in the additional traits assessed. Although QTL detection for the latter traits could be lower, the occurrence of NPLS might be less influenced by them.

QTLs for NPLS

Three QTLs for NPLS were identified, explaining nearly one-half of the genotypic variance in the DH population derived from the cross IPZ24727/Barke. As no QTLs for NPLS resistance have been published to date, the comparison was made with QTLs or genes of different kinds or expressions of abiotic stress. The exact positions of published QTLs or genes remain to be determined in the population map due to the lack of anchor markers on some of the genomic regions. Nevertheless, the QTL on chromosome 1H might be located in the vicinity of *nec1* (Lundqvist et al. 1997), and the *Hv-HIR1* and *Hv-HIR2*-genes (Rostoks et al. 2003) could be related with QTLs for NPLS on chromosome 4H bin 10 and on chromosome 5H near the centromere. The QTL for NPLS on chromosome 5H has not been mentioned previously as it appeared only in 2000, but it has a high explained phenotypic variance (data not shown). Therefore, that QTL might be explained by QTL×E interactions (Groh et al. 1998).

All NPLS resistance alleles were inherited by the resistant parent IPZ24727, and no epistasis was determined. Mainly additive gene action was involved in NPLS resistance and no transgressions were observed for NPLS in the DH lines.

The most important QTL detected for NPLS was located on chromosome 4H near the *mlo* locus. QNpls.lfl-4H showed the highest LOD score (38.6), explained phenotypic variance (34%) and additive effect (4.3) and might be classified as a major gene. Moreover, there are several authors that have mapped genes or QTLs for different traits in the target region on chromosome 4H bin10 (Kleinhofs 2003) that showing large influences on several plant characteristics and resistances.

The QTL QNpls.lfl-1H had rather low values of LOD and explained phenotypic variance and additive effects. The latter was determined in every scored leaf level and environment, reaching its highest effect in 2002. The QTL QNpls.lfl-7H was mapped together with QTLs for powdery mildew resistance, plant height and TKW and had rather a low significance.

Breeding application for NPLS resistance in spring barley

The QTLs mapped for NPLS are a first step towards the development of cultivars with an improved resistance. After selection of the best markers, validation of the QTLs for NPLS resistance on the basis of marker-assisted backcross lines should be carried out. Thereafter, it is advisable to test the selected markers in other genetic backgrounds in order to confirm their selection response and their further use in the development of new resistant varieties.

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