Septoria tritici blotch resistance in wheat: Identification and localisation with DNA markers

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Abstract

The disease septoria tritici blotch of wheat is caused by the fungus *Mycosphaerella graminicola* (anamorph *Septoria tritici*). This disease is currently among the most important in European wheat areas. Resistance to septoria tritici blotch is consequently one of the important traits to consider when breeding wheat cultivars for the European market. To increase selection efficiency and to allow selection for resistance in breeding material in years with unfavourable conditions for disease development, molecular markers for resistance to septoria tritici blotch are being developed. A doubled haploid population was produced from a cross between the susceptible cultivar Savannah and the resistant cultivar Senat. Parents and progeny were tested in growth chamber and field with isolates of *M. graminicola*. In growth chamber, Senat was resistant to the Dutch isolate IPO323 and to the Danish isolate Risø97-86, whereas Savannah was susceptible to both isolates. Segregation of resistance to IPO323 in the progeny was in agreement to a 1:1 ratio. Using Bulked Segregant Analysis, three microsatellite markers linked at a distance of 2-15 cM from the gene conferring resistance to IPO323 was identified. Resistance to Risø97-86 followed a 1:3 ratio of resistant to susceptible lines, indicating either the involvement of two complementary genes or the presence of a suppressor, which is able to suppress resistance to Risø97-86. A preliminary QTL analysis showed that both the resistance gene conferring resistance to IPO323 and the microsatellite markers linked to this gene had a significant effect on resistance in the field. A further two microsatellites closely linked (2.4 cM) on chromosome 2D also had a significant effect on field resistance.

Introduction

Septoria tritici blotch is an important fungal disease of wheat, caused by the ascomycete *Mycosphaerella graminicola* (anamorph *Septoria tritici*). In Europe, the importance of this disease has increased over the last 20 years. Increased susceptibility of the cultivars grown has been suggested a contributing factor (BAYLES 1991).

The pathogen infects leaves of the wheat plant. Here it produces elongated necrotic blotches with black fruit bodies after a relatively long latent period of more than 3 weeks (SHAW, 1990). These fruit bodies are pycnidia producing the asexual pycnidiospores. After a further period of 5-12 weeks pseudothecia holding the sexual ascospores appear (ERIKSEN 2000, HUNTER et al. 1999). Pycnidiospores are dispersed over short distances with rain splash, whereas ascospores are dispersed by wind over longer distances. In a crop of winter wheat, the majority of primary infections in autumn are probably initiated by ascospores blown in from nearby fields of stubble, perhaps more than 70 spores per square meter initiate these infections (SHAW & ROYLE 1989, ZHAN et al. 2001). There are reports that ascospores occur throughout the crop-growing season (ERIKSEN 2000, HUNTER et al. 1999). However, the importance of ascospores for the disease development over spring and summer is probably limited. During this period pycnidiospores contributes more to disease development primarily due to the shorter period from infection to sporulation for pycnidia compared to pseudothecia (ERIKSEN et al. 2001).

Examples of resistant cultivars becoming susceptible to the local *M. graminicola* population after a few years does exist (COWGER et al. 2000), although septoria tritici blotch resistance have been thought to remain relatively stable over years (JOHNSON 1992). The fairly high frequency of sexual reproduction in populations of *M. graminicola* could result in rapid loss of the effectiveness of resistance in new resistant cultivars due to recombination of genes for virulence and aggressiveness in the pathogen population. Mathematical modelling of the frequency of pathotypes adapted to certain hypothetical cultivars suggested that sexual reproduction will increase the rate of adaptation in the pathogen population, but the modelling also suggested that the effect is likely to be very small (ERIKSEN 2000).

Single dominant genes, as well as recessive genes control resistance to *M. graminicola*, and additive modes of inheritance have also been found (ROSIELLE & BROWN 1979, SOMASCO et al. 1996, WILSON 1985, ZHANG et al. 2001). Only few examples of major gene resistances to *M. graminicola* used in breeding exist (EYAL 1999). Five genes for resistance to *M. graminicola* have been described and named *Stb1*, *Stb2*, *Stb3*, *Stb4* and *Stb5* (ARRAIANO et al. 2001, SOMASCO et al. 1996, WILSON 1985). The chromosomal location of one of these genes, *Stb5*, has been determined. This gene is located on the short arm of chromosome 7D near the centromere (ARRAIANO et al. 2001). Chromosome 3A has been identified as carrying genes for resistance to the Dutch *M. graminicola* isolate IPO323 (ARRAIANO et al. 1999). Several chromosomes in two wheat cultivars, a synthetic hexaploid wheat and a *Triticum spelta* genotype were reported to carry resistance genes to two Argentinean *M. graminicola* isolates (SIMON et al. 2001).

In wheat breeding there is an increasing interest in using molecular markers to aid in selecting for traits, which can otherwise be difficult to select for. Resistance

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to *M. graminicola* is a difficult trait, because the disease is highly dependent on environmental conditions (SHAW & ROYLE 1993), and because of limited knowledge about the inheritance of resistance in breeding material. An important part of identifying a new *M. graminicola* resistance gene is thus to establish the location of the gene on the chromosomes, and develop a molecular marker that can be used for screening breeding lines. The objective of this study was to provide linked molecular markers for resistance to *M. graminicola*, for application in wheat breeding. The approach taken was mapping of resistance genes effective toward single isolates in growth chamber tests, combined with QTL analysis on field data.

Materials and methods

Plant material

Using the wheat x maize method, a population of 76 doubled haploid lines was produced from the F1 generation of a cross between the winter wheat cultivars Savannah and Senat. The cultivar Senat possesses qualitative resistance and a high level of quantitative resistance in the field to *M. graminicola*, whereas the cultivar Savannah is highly susceptible to *M. graminicola*.

Growth chamber test

Test of parents and progeny of the Savannah x Senat cross and a number of additional cultivars with single spore isolates was performed in growth chamber. Ten seeds were sown in a line next to a net. After ten days the fully developed primary leaves were placed in horizontal position by fixation to the net with a piece of string, and with the adaxial side of the leaves facing upwards. Inoculum of *M. graminicola* was produced by growing the fungus in liquid yeast glucose medium for 5 to 8 days. The seedlings were inoculated by applying a spore suspension of 10^6 spores/ml to the horizontally fixed leaves. The pots were subsequently kept at high humidity in bags for 48 hours. Growth chamber conditions were 20°C with 18 hours of light. Assessment of disease severity as percent coverage with necroses and pycnidia was performed 21-22 days after inoculation.

Field experiment

A field experiment was established with the parents and lines of the progeny population grown in two rows of 1 m. Due to lack of seed only 71 of the lines were included and replicates were only possible for half the lines of which three replicates were sown. The experiment was inoculated twice after the flag leaves were fully expanded with a mixture of 11 *M. graminicola* isolates. These isolates had previously been shown to be virulent on Senat when tested in growth chamber. Disease severity was assessed as percent coverage of flag leaves with lesions bearing the fruit bodies of *M. graminicola*. This field experiment will be replicated on two locations in the 2001/02 season, with a total of 110 DH-lines.

Molecular marker analysis

DNA of parents and progeny was extracted using the CTAB method. The molecular markers used were microsatellite markers developed by RÖDER et al. (1998) and the Wheat Microsatellite Consortium (WMC). In the Wheat Microsatellite Consortium, Clones containing microsatellites from a microsatellite enriched genomic library (EDWARDS 1996) were sequenced by members of the consortium and PCR primers were designed by Agrogene.

PCR reactions were performed on a Perkin Elmer 9700 thermal cycler. Cycling conditions were, 1 min. denaturation at 94°C, 1 min. annealing at 50, 55 or 60°C (depending on the microsatellite) and 1 min. extension at 72°C, for 31 cycles. The samples were initially denatured for 5 min. and the cycling was followed by an extension step of 7 min. The PCR reaction mix contained 0.25 µM of each

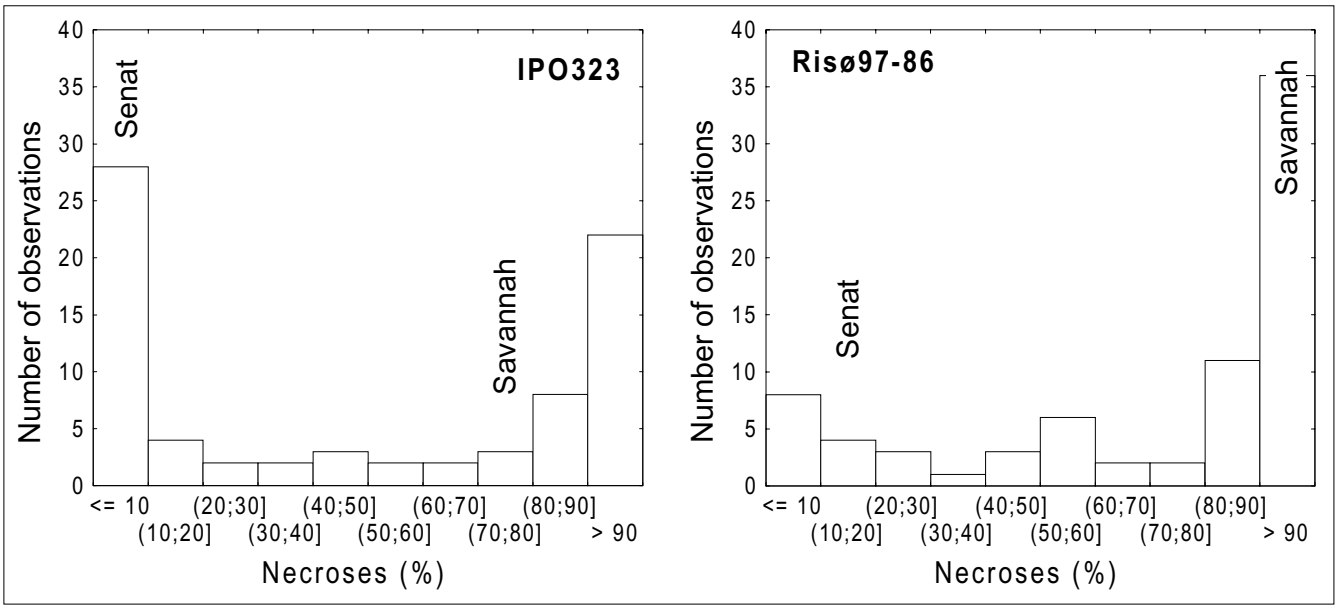

Figure 1. **Histograms showing the results of the disease tests on the doubled haploid progeny of the cross Savannah x Senat in growth chamber. A, Average leaf coverage with necroses on seedlings inoculated with *Mycosphaerella graminicola* isolate IPO323. B, Average leaf coverage on seedlings inoculated with *M. graminicola* isolate Risø97-86. The disease reaction of the parents is indicated on the graphs.**

Table 1: **Segregation, based on growth chamber experiments, of specific resistance to the *Mycosphaerella graminicola* isolates IPO323 and Risø97-86 in the doubled haploid population from the cross Savannah x Senat. Deviation from the expected segregation ratio was tested with the chi-square test (χ^2). NS = not significant.**

Isolate	Observed number Resistant	Susceptible	Expected ratio	χ^2 (P)
IPO323	36	40	1:1	0.64^{NS}
Risø97-86	16	59	1:3	0.46^{NS}

primer, 1.5 mM $MgCl_2$, 200 µM of each deoxynucleotide, 1x Taq buffer, 0.5 unit Taq polymerase and 50 ng of template DNA, in a reaction volume of 10 µl. The fragment length of amplified microsatellites was determined on an ABI377 automatic DNA sequencer, using fluorescently labelled primers.

Bulked segregant analysis (BSA) (MICHELMORE et al. 1991) was performed to identify polymorphic markers. Markers showing polymorphism in the BSA analysis were tested on the whole population, and linkage to the resistance gene was calculated using the program Mapmaker version 3.0 (LANDER et al. 1987). QTL analysis was performed on field data using the nonparametric mapping procedure of the program MapQTL version 3.0 (Van OIJEN & MALIEPAARD 1996).

Results

Growth chamber test

In total 16 *M. graminicola* isolates collected in Denmark and one Dutch isolate IPO323, obtained from G.H.J. Kema, Plant Research International, Wageningen, The Netherlands, were tested on Savannah and Senat in growth chamber. Five isolates were avirulent on Senat and all 17 isolates were virulent on Savannah. Two of the avirulent isolates IPO323 and Risø97-86 gave a particularly good differentiation between Savannah and Senat. These two isolates were found to differ in their reaction on the cultivar Flame, IPO323 being avirulent and Risø97-86 being virulent. The isolates IPO323 and Risø97-86 were tested on the progeny of the cross Savannah ´ Senat (*Figure 1*). Lines with coverage of necroses of less than 40% in growth chamber tests were classified as resistant. However, some lines with less than 40% necroses had pycnidial coverage of more than a few percent, these lines were considered susceptible. Some lines with slightly more than 40% necroses produced no pycnidia. These lines were rated as resistant. Segregation for resistance to IPO323 was in agreement to a 1:1 ratio, and resistance to Risø97-86 did not differ significantly from a 1:3 ratio of resistant to susceptible lines (*Table 1*).

The segregation data suggested, that one gene is responsible for resistance to IPO323, and that two genes are involved in resistance to Risø97-86. Lines resistant to Risø97-86 were also resistant to IPO323, whereas lines resistant to IPO323 were not necessarily resistant to Risø97-86.

Table 2: **QTL analysis on data from a field trial with the Savannah x Senat population artificially inoculated with 11 *Mycosphaerella graminicola* isolates. Kruskal-Wallis statistic from MapQTL for the effect of five microsatellite loci and the gene providing resistance to *Mycosphaerella graminicola* isolate IPO323 is shown. Last column gives the proportion of the phenotypic variance explained by *Xgwm369* and *Xwmc144* from a multiple regression analysis.**

Chromosome	Locus	Genetic distance (cM)	K	Kruskal-Wallis statistic Level of significance	Variance explained (%)
3A	*Xgwm369*	1.5	14.4	P < 0.0005	18
	Resistance gene	-	12.9	P < 0.0005	
	Xwmc60	14.6	7.4	P < 0.01	
	Xwmc50	15.4	6.2	P < 0.05	
2D	*Xwmc18*	-	10.4	P < 0.005	
	Xwmc144	2.4	10.6	P < 0.005	14

Mapping of resistance

Bulks of DNA from resistant and susceptible plants were generated on the basis of growth chamber test with the *M. graminicola* isolate IPO323 and results from the field, such that only plants for which growth chamber and field results agreed were included. In total, 60 microsatellite markers were screened on parents and bulks. Five microsatellites *Xgwm369*, *Xwmc18*, *Xwmc50*, *Xwmc60* and *Xwmc144* showed polymorphism in the BSA analysis. The microsatellite markers *Xgwm369*, *Xwmc50* and *Xwmc60* were linked 1.5, 15.4 and 14.6 cM respectively from the resistance gene providing resistance to IPO323 (*Figure 2*). The markers *Xwmc18* and *Xwmc144* were not linked to this resistance gene. *Xgwm369* and *Xwmc50* maps to chromosome 3A in the ITMI (International Triticeae Mapping Initiative) population, and *Xwmc18* and *Xwmc144* to chromosome 2D (RÖDER et al. 1998, M. J. CHRISTIANSEN pers. comm.).

QTL analysis

The field experiment was assessed at growth stage 69 (BBCH scale), 27 days after the last inoculation. The data from the field experiment showed a more continues distribution than the growth chamber data with single isolates, and there was no clear segregation into susceptible and resistant lines (*Figure 3*). A preliminary QTL analysis was performed with the program MapQTL. Nonparametrical Kruskal-Wallis analysis was chosen due to the few markers available making interval mapping inappropriate. The microsatellite markers *Xwmc18* and *Xwmc144* on chromosome 2D and *Xgwm369*, *Xwmc50* and *Xwmc60* and the resistance gene locus for resistance to IPO323 on Chromosome 3A (*Figure 2*), all showed a significant effect on resistance in the field (*Table 2*). The putative QTL alleles providing resistance came from Senat on both chromosome 2D and 3A. The level of significance for the putative QTL on chromosome 3A increased from *Xwmc50* toward the resistance gene locus. A stepwise multiple regression analysis was performed on logit transformed data. The optimal model included the microsatellite markers *Xgwm369* and *Xwmc18*, with a level of phenotypic variance explained of 18% and 14% respectively.

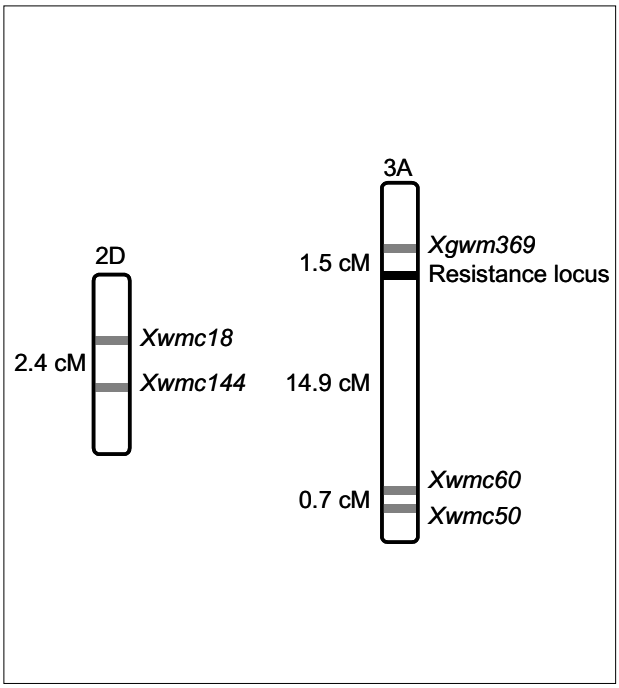

Figure 2: **Partial map of wheat chromosomes 2D and 3A showing the positions of microsatellite markers and the locus for resistance to *Mycosphaerella graminicola* isolate IPO323.**

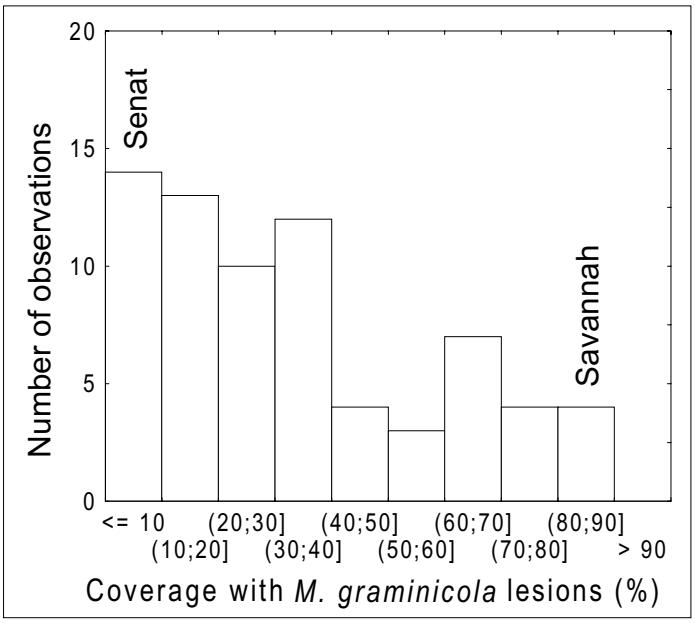

Figure 3: **Histogram showing percent coverage with *Mycosphaerella graminicola* lesions on flag leaves of the doubled haploid progeny of the cross Savannah x Senat in a field trial. The trial was inoculated with a mixture of 11 isolates of *M. graminicola*. The disease reaction of the parents is indicated on the graph.**

Verification of DNA marker in different cultivars

A number of commercial cultivars and cultivars with previously described septoria tritici blotch resistance genes, were tested with the microsatellite marker *Xgwm369* and the *M. graminicola* isolates IPO323 and Risø97-86 (*Table 3*). The marker allele of 300 base pairs (C) present in Senat was also present in Hereward, Kris, Stakado and Cleopatra 74. These five cultivars were resistant to IPO323, but differed in resistance to Risø97-86 to which Senat and Stakado were resistant but Hereward and Kris susceptible. Two cultivars, Flame and Tadorna possessed the B allele and were both resistant to IPO323, and susceptible to Risø97-86. The remaining seven cultivars were all susceptible to IPO323 and to Risø97-86, these cultivars possessed the A allele, apart from Wasmo which had a null allele.

Discussion

Specific resistance to *M. graminicola*

The specific resistance to the Dutch *M. graminicola* isolate IPO323 in the cultivar Senat, seem to be controlled by a single gene located on chromosome 3A. Several cultivars show specific resistance to IPO323 (BROWN et al. 2001, *Table 3*) and resistance to IPO323 has, in agreement with the present study, been located to chromosome 3A in the cultivar Bezostaya 1 (ARRAIANO et al. 1999). Furthermore, single gene inheritance of resistance to IPO323 has been suggested for resistance in the cultivar Flame (BRADING et al. 1999). Avirulence in IPO323 towards a number of cultivars is controlled at a single locus in the fungus (KEMA et al. 2000), and it has been suggested that a gene-for-gene relationship exist between this isolate and the resistance gene in the cultivar Flame (BRADING et al. 1999).

Resistance of Senat to Risø97-86 apparently involves two genes one of which is the IPO323 resistance gene, and is not in agreement with the gene-for-gene relationship. This is concluded from the 1:3 segregation of resistant to susceptible lines, and the fact that only lines resistant to IPO323 were resistant to Risø97-86. Among the additional cultivars tested with *Xgwm369* and Risø97-86, one cultivar Stakado was resistant to Risø97-86. This cultivar possessed the same marker allele (C) at the *Xgwm369* locus as Senat. Another two cultivars with this allele were susceptible to Risø97-86, again indicating that a gene at an additional locus must be involved in resistance to this isolate. This result can be explained by either two complementary genes providing resistance to Risø97-86 or by a suppressor, which is able to suppress the expression of resistance to Risø97-86. The latter interpretation would imply that the cultivars Senat and Stakado possess the resistance gene but not the suppressor and are consequently resistant to both IPO323 and Risø97-86. Savannah possesses the suppressor because the suppressor segregates in the Savannah x Senat progeny. Flame, Hereward and Kris posses both the suppressor and the resistance gene, because they express resistance to IPO323 but not to Risø97-86. Genes suppressing the expression of resistance genes have been identified in a number of cases. It has been observed that suppressers on the D genome of hexaploid wheat can suppress leaf rust and stem rust resistance introgressed from wild relatives (BAI & KNOTT 1992, KERBER & GREEN 1980). Suppressors have been identified on the AB and D genomes, suppressing stripe rust resistance in synthetic hexaploids (KEMA et al. 1995).

Furthermore, expression of the powdery mildew resistance gene *Pm8* carried

Table 3: **Alleles of the microsatellite *Xgwm369* and reaction to *Mycosphaerella graminicola* isolate IPO323 and Risø97-86 for 10 commercial winter wheat cultivars, and four cultivars with the previously described septoria tritici blotch resistance genes *Stb2* and *Stb4*.**

Cultivar	Reaction to IPO323[1]	Marker allele *Xgwm369*[2]	Reaction to Risø97-86[1]
Wasmo	S	Null allele	S
Ritmo	S	A	S
Savannah	S	A	S
Solist	S	A	S
Terra	S	A	S
Nova Prata (*Stb2*)	S	A	S
Veranopolis (*Stb2*)	S	A	S
Tadorna (*Stb4*)	R	B	?
Flame	R	B	S
Hereward	R	C	S
Kris	R	C	S
Senat	R	C	R
Stakado	R	C	R
Cleopatra 74 (*Stb4*)	R	C	?

[1] S: susceptible and R: resistant
[2] Fragment lengths of alleles in base pairs A: 189bp, B: 197bp, and C: 300bp

on the wheat- rye translocation T1BL•1RS, is suppressed by a dominant suppressor in the wheat genome (HANUSOVA et al. 1996). It has been suggested that suppressors can be specific for resistance genes (BAI & KNOTT 1992, KEMA et al. 1995), such that one suppressor suppresses the expression of a specific resistance gene but not other resistance genes present in the genome. In the present investigation resistance to a specific isolate (Risø97-86) seem to be suppressed by a suppressor, whereas resistance to another isolate (IPO323) was not affected, even though the same gene in Senat apparently provides resistance to both isolates. Alternatively, a gene closely linked to the IPO323 resistance gene could be providing resistance to Risø97-86, with the suppressor gene from Savannah specifically suppressing this resistance gene. The Savannah x Senat mapping population of 76 lines could be too small to detect this.

Resistance to M. *graminicola* in the field

Among the isolates tested in growth chamber in this study, only 4 out of 16 (25%) isolates collected in Danish wheat fields, were avirulent on Senat. It would thus seem that the resistance possessed by Senat has broken down. This is in contrast to what is seen under field conditions, where the resistance of Senat is highly effective, indicating a high level of adult plant resistance. The QTL analysis on the field data was preliminary, only data for five markers and disease data from one year and one location was available. The microsatellite locus *Xgwm369* showed a slightly higher level of significance in the QTL analysis than did the resistance gene providing resistance to IPO323 in growth chamber. This probably reflects that marker genotyping is more reliable than disease testing. Consequently, the QTL analysis suggests that there is an effect of the IPO323 resistance gene in the field against an inoculated *M. graminicola* population. In spite of this gene being ineffective against the individual isolates of the population in growth chamber tests. QTL loci, mapping to approximately the same location as specific resistance genes have previously been reported, e.g. for powdery mildew and leaf rust of barley (BACKES et al. 1996, KICHERER et al. 2000). The putative QTL on chromosome 3A explained 18% of the phenotypic variation in field resistance, and the putative QTL on chromosome 2D explained 14% of the phenotypic variance. Attempts to identify and map further QTLs as well as more precisely locating the putative QTL on chromosome 2D will be made using data from additional trials with 110 DH-lines and a more complete map with more microsatellite and AFLP markers.

Marker assisted selection

Apart from the C allele of *Xgwm369* another allele B, present in the cultivars Flame and Tadorna, was associated with resistance to IPO323. None of the susceptible cultivars were found to possess either the B or the C allele. As the marker is not absolutely linked false positives will occur at a low frequency when using it for marker assisted selection. The QTL analysis verified that the resistance gene detected in growth chamber was also effective in the field, a necessary condition if the marker is to be used for marker assisted selection. Identification of markers for QTLs will make selection for favourable QTL alleles more efficient. It has been shown by simulation, that markers for QTLs can be used to increase the effectiveness of selection for quantitative traits over that of phenotypic selection (CHARMET et al. 1999). CHARMET et al. (1999) used marker information to select pairs of doubled haploid lines to be intercrossed in order to accumulate the maximum amount of favourable QTL alleles. However, the accuracy of the QTL position had a big impact on the selection efficiency.

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