

## Selection of AFLP Markers Linked with Crown Rust Resistance in *Lolium*: Efficiency of Bulk Segregant Analysis in an *Allogamous* Species

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### Abstract

Ryegrasses are important forage species. An important foliar disease in *Lolium* is crown rust, caused by the fungus *Puccinia coronata*. This cosmopolitan biotrophic fungus causes forage and seed losses, and decoloration of amenity grasslands. In breeding towards resistant cultivars, it is important to know the mode of inheritance of crown rust resistance. A pair cross between a susceptible and a resistant plant resulted in a F<sub>1</sub> population segregating for rust resistance. According to the segregation data, major genes were assumed to encode for crown rust resistance. In a bulk segregant analysis (BSA) 187 primer combinations revealed AFLP markers linked with resistance. Two groups of genetically unlinked markers were identified, explaining together 35% of the phenotypic variation.

**Keywords:** *L. perenne*; *Puccinia coronata*; BSA; molecular markers

### INTRODUCTION

On at least 12 million ha in Europe, ryegrass cultivars (*Lolium* spp.) are used (<http://europa.eu.int/comm/agriculture/>). They are important forage species, used mainly as pasture, hay crop or ground cover (PRINE 1990). To reduce the losses chemical control can be used, but in sustainable agriculture, especially for forage crops, chemical control of pathogens is not desirable. Good management practices which reduce conditions favourable to crown rust constitute the best method to reduce fungal epidemics. The use of resistant cultivars is one of them (THOMAS 1991). Therefore improving genetic resistance is a major goal in most ryegrass breeding programs.

The advent of molecular marker technologies enhanced the genetic dissection of complex traits. They are powerful tools since they allow disease resistances to be broken up into their components by quantitative analysis and quantitative trait locus mapping (CHANTRET *et al.* 2000). Markers permit marker assisted selection (MAS), which is an attractive approach when pathological tests in conventional

breeding are costly, time-consuming and dependent on environmental conditions (CHANTRET *et al.* 2000).

Bulk Segregant Analysis (BSA) is the classical way to find genetic markers linked to a trait under monogenic control (MICHELMORE *et al.* 1991). The underlying principle of BSA is the grouping of the informative individuals together so that a particular genomic region can be studied against a randomised genetic background of unlinked loci. In this study, the use of BSA to identify AFLP markers linked to crown rust resistance in a full sib *L. perenne* family is discussed.

### MATERIALS AND METHODS

#### Plant material and infection tests

*L. perenne* accessions were screened for crown rust resistance at three different field locations in 1997. A resistant and a susceptible genotype were selected and crossed in the greenhouse under controlled conditions. When seeds were ripe, stalks were harvested. Plants were tested for crown rust resistance using

the artificial infection test described by ADAMS *et al.* (2000). The first infection was carried out when plants were 6 weeks old and was repeated 2 times at intervals of 1 month, using the same plants.

### Bulk segregant analysis

AFLP data was generated as described by ROLDÁN-RUIZ *et al.* (2000). For each population, resistant (R) and susceptible (S) bulk samples were constructed by pooling equal amounts of 10 preamplified DNAs. Three bulks were made: two R bulks and one S bulk. The first R bulk consisted of 10 R plants harvested on the R parent and another bulk consisted of 10 R plants harvested on the S parent, all with mean rust score of 1. S bulks consisted out of 10 plants having a mean score  $> 1 + 2 \times \text{SD}$  of the population. The primer combinations (PC) yielding candidate markers were subsequently applied to all bulk individuals and further to 252 plants of the population to confirm linkage.

### Statistical analysis

As the data generated in this BSA analysis are discrete binary data, conventional statistics can not be used, in strict statistical terms. In order to increase the power of the tests, permutation tests were designed by MOERKERKE *et al.* (Gent University, pers. commun.). For each candidate marker, the original data set was subjected to linear regression, resulting in an original  $T_0$  statistic. Marker data were permuted 800 times, and for each permuted data set the associated T statistic was calculated. The distribution of the obtained T-values was constructed and the percentiles of the distribution were calculated. The  $H_0$  hypothesis (no association between marker and rust score) was rejected if the original T ( $T_0$ ) was outside the 2.5% and the 97.5% percentiles of the permutation distribution ( $\alpha = 5\%$ ). Simple and multiple linear regression was used to calculate the coefficient of determination,  $R^2$ , providing a measure of the proportion of the total variance explained by each retained marker or set of markers. Map distances between markers were calculated using the linkage software Joinmap version 3.0 (VAN OOIJEN & VOORIPS 2001).

## RESULTS

A high degree of consistency between the rust score assigned to single plants at different rounds of artificial inoculation was observed. This impression

was confirmed by the significant correlation values obtained between scores of different infection rounds. The correlation coefficient of the rust score of round 1 and 2 is 0.581, of round 1 and 3 is 0.535 and of round 2 and 3 0.681. They are all significant at the 0.01 level. The frequency distribution of the mean rust score in the  $F_1$  population is given in Figure 1. As *Puccinia* spp. are biotrophic fungi, we expect to find genes that display gene-for-gene specificity (WISE *et al.* 1996). The frequency distributions of the studied population is a skewed normal distribution (Figure 1). The skewness is towards the score of the R parent.

AFLP was used as marker technique in the BSA analysis. Parents and bulks were screened using in total 187 PC. On average 35 AFLP fragments/PC were polymorphic between the parents. This high amount of polymorphisms can be explained by the heterozygosity and heterogeneity of the parent plants. This means, that 187 PCR reactions, generating 187 AFLP fingerprints, screened 6 545 polymorphic markers. The average number of candidate markers (polymorphic in the parents and in the bulks) was 1.4 markers/PC. In a second screening, the most promising polymorphisms were confirmed by testing their behaviour in all the individuals used to construct the bulks. A marker linked with R was retained when it was present in less than 40% of the S bulk individuals and present in more than 60% of the R bulk individuals. Other markers were classified as random segregating markers. In total, 40 markers were tested, of which 20 were retained. Out of these, 4 PC amplifying 6 potentially linked markers were chosen to be tested on all the individuals of the population. Permutation tests identified significant

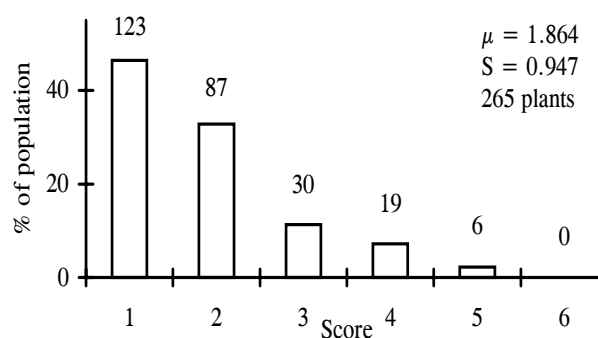


Figure 1. Frequency distribution of mean rust scores of the studied population. Score 1 refers to resistant plants and score 5 to susceptible plants. The number of plants representing the percentage are noted on top of the bar. The population mean, the standard deviation and the number of plants in the population is given in the box

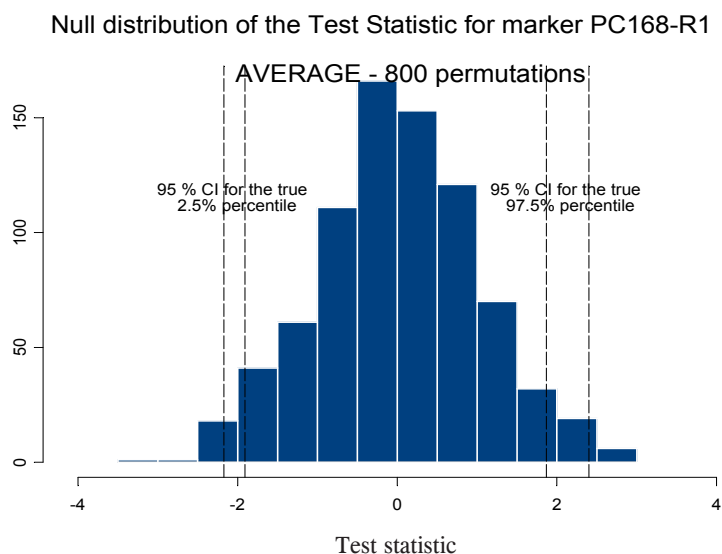


Figure 2. Test for significant association of marker PC168R1 with rust resistance using permutation tests. 2.5 and 97.5% percentiles of the distribution of the Test statistics obtained during 800 permutations. The original test statistic is  $-7.981$  and is situated in the 2.5% percentile of the distribution, indicating the significant association of the marker with rust resistance

association between the 6 selected markers and crown rust resistance. F.e. for the PC168R1 marker the  $T_0$  test statistic ( $-7.981$ ) is situated in the 2.5% percentile of the distribution of the simulated  $t$ -test statistics (Figure 2). To estimate the proportion of variance in the crown rust data explained by each marker, a linear regression model was fitted to the data. The coefficient of determination  $R^2$  was used as a measure of the magnitude of the marker-associated phenotypic effect (CHAGUÉ *et al.* 1997).  $R^2$  values of more than 20% were found (PC106R2 25.1% and PC168R1 21.3%). Multiple linear regression of the association of the 6 markers and resistance resulted in  $R^2$  of only 35%.

## DISCUSSION

Out of the skewed normal distribution of the rust score we can conclude that several major genes are involved in resistance. The non-discrete nature of the distribution can be explained by the presence of minor genes which influence the action of the major genes.

The use of AFLP as a marker technique in combination with BSA analysis was a good choice as AFLP primer combinations revealed a high number of potentially linked markers with the studied trait. The efficiency of a BSA to identify potentially linked markers can be measured by the frequency at which markers, polymorphic in the parent plants, show differences between bulks. Under our conditions, we screened 6 545 polymorphic markers. If we consider that AFLP markers are distributed uniformly

throughout the genome, that the length of the *Lolium* genome is 1050 cM (HAYWARD *et al.* 1998) and that we are looking for a marker within a window of 10% recombination either side of the target locus, we expect 1.90% of the polymorphic loci to fall within a window of 20 cM around the target locus, and to be linked to the target gene. The percentage observed in this study was 4.07%. Several factors can contribute to the detection of more candidate markers than theoretically expected. A reason can be the high level of heterozygosity and heterogeneity in the parent plants and the fact that in this specific kind of cross more false positives are expected (the chance to select a marker, while not linked to the trait is  $2 \times 10^{-3}$ ). This is higher than in a BSA analysis on a  $F_2$  population generated from two homozygous plants. As only 50% of the initial selected markers were retained after the second screening, our suspicion that most of the markers initially selected by the BSA analysis (see above) were false positives is confirmed.

The observation that the combination of loci results only in a small increase in the proportion of phenotypic variance explained ( $R^2$ ), indicates that some selected loci are linked. Linkage analysis determined two clusters of markers: PC026R3 and PC026R4 are tightly linked (6.03 cM), and PC106R2 and PC168R2 are tightly linked (9.05 cM).

To explore the possibility of using the markers that display a significant effect for MAS, the differences in rust score in the subpopulations with and without the marker were analysed. By using the presence/absence of marker PC106R2 as criterion to subdivide the population, an improvement of 0.48 units (rust

score) was achieved with respect to the average score of the complete population (1.86), indicating the potential of this marker for MAS.

Although we were able to identify genomic regions involved in rust resistance, the total percentage of the phenotypic variance explained by these loci is still rather low. The undetected QTLs in the current study may result from the limitation of the BSA strategy because this technique may directly target the resistance QTL with major effects, not those with minor effects. A QTL analysis will help us identify those other genomic regions involved in resistance.

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