GENETIC ANALYSIS OF INHERITANCE OF PARTIAL RESISTANCE TO FUSARIUM *OXYSPORUM* IN ASIATIC HYBRIDS OF LILY USING RAPD MARKERS

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Abstract

Linkage of RAPD markers with loci involved in partial resistance to *Fusarium* in Asiatic hybrid lilies was investigated. Variation in resistance was found in two greenhouse tests using scale bulblets of 150 descendants of a backcross population. The progeny did not show a clear Mendelian segregation in *Fusarium* resistance. Three out of 213 RAPD markers were significantly ($p < 0.005$) linked to *Fusarium* resistance explaining approximately 24 percent of the total phenotypic variance of the resistance. The construction of a genetic map with all RAPD markers was hampered because of different segregation types due to the dominant marker system and the low number of descendants evaluated per RAPD marker. Genomic regions, where *Fusarium* resistance loci were calculated to be linked to markers, were constructed. The use of RAPD markers for selection of quantitative traits and construction of linkage maps is discussed.

Keywords

Fusarium oxysporum, inheritance, *Lilium,* RAPD markers, partial resistance, quantitative trait loci.

1. Introduction

The lily *(Lilium* L.), a vegetatively propagated perennial crop, is one of the economically most important flower bulbs. The soil-borne pathogen *Fusarium oxysporum* f.sp. *lilii* Imle causes basal rot in lily and threatens bulb cultivation seriously. Resistant cultivars can play an important role in the prevention of damage and in the reduction of the application of fungicides to prevent further environmental pollution. Variation in partial resistance to *Fusarium* has been described for several cultivars and *Lilium* species, but so far absolute resistance has never been reported (Imle, 1942a; Imle, 1942b; Straathof & Van Tuyl, 1994). In order to select for new *Fusarium* resistant lily cultivars, screening tests at clonal level (Straathof & Löffler, 1994a) and seedling level (Straathof & Löffler, 1994b) have been developed. Screening tests at clonal level can only be performed several years after crossing. Seedlings can be tested in the first year after sowing. Selecting seedlings resulted in a positive selection response but because of environmental variation retesting at clonal level is necessary. The mode of inheritance of *Fusarium* resistance in Asiatic hybrids of lily has not been deciphered yet, but diallel analysis of seedlings suggests that several genes are involved (Straathof & Löffler, 1994b).

Molecular markers have been recognized as possible tools for indirect selection on traits, independently from environmental variation. They can be used to speed up selection (e.g. at seedling stage) and for genetic studies in crops with a long juvenile period and/or slow propagation rates (e.g. flower bulbs). For a review on potential uses of molecular

markers, see Gebhardt & Salamini (1992) or Tanksley (1994). In flower bulbs, no reports of the use of molecular markers for indirect selection have been reported so far.

1.1. RAPD analysis

Scales and scale bulblets were used for total DNA isolation according to either a tomato leaf extraction procedure (Van der Beek et al., 1991) or a method based on the chemical disclosure of tissue with sodium ethyl-xanthogenate (Jhingan, 1992). DNA concentrations were determined using a fluorometric assay with Hoechst 33258 in a Hoefer TKO 100. DNA was diluted to 10 ng per μl in TE buffer, and stored in aliquots at -20 °C until use.

RAPD reactions were conducted in reaction mixtures containing 25-50 ng total DNA, 50 ng 10-mer primers (Operon technologies), 200 μM of each dNTP and 1 unit Amplitaq DNA polymerase or 2 units Amplitaq DNA polymerase Stoffelfragment (Perkin Elmer). RAPD reactions were performed in either a Perkin Elmer DNA thermal cycler 480 [40 cycles of denaturation at 92 °C (1 min), annealing at 35 °C (1 min) and extension at 72 °C (2 min)] or a Perkin Elmer GeneAmp PCR system 9600 [40 cycles 94.3 °C (24 sec), 35 °C (20 sec) and 72 °C (74 sec)]. In order to detect polymorphisms, the 10-mer primers were pre-tested on the three (grand)parents of the population.

RAPD products were stained with ethidium bromide after electrophoresis on 1.5 % agarose gels in 1 x TBE according to Sambrook et al. (1989). Polymorphisms present in the resulting banding patterns were scored from photographs of the agarose gels, recorded in data matrices and used for statistical analysis.

1.2. Statistical analysis

Disease ratings were analysed according to a threshold model for ordered categorical data (McCullagh, 1980; Jansen, 1990), using a probit link function. For each clone within an experiment, a disease severity score (DSS) was calculated by the threshold model. This score may be considered as a transformed average value of the disease ratings of each clone on an underlying linear scale (Straathof et al., 1993). Conclusions concerning block and genotype effects were assessed by analysis of deviance (McCullagh & Nelder, 1989); deviances were compared with the table of chi-squared distribution.

The heritability of *Fusarium* resistance was calculated as $h^2 = \sigma^2_g / (\sigma^2_g + \sigma^2_e/n)$, with σ^2_g = expected genotypic variance, σ^2_e = expected residual variance, and n = the number of plants per genotype.

Association of an individual segregating RAPD marker with either the DSS values of 1992 or 1993 was assessed by applying Kruskal-Wallis rank-sum test (see e.g. Lehman, 1975), as implemented in the MapQTL computer program (J.W. Van Ooijen, *personal communication).* The Kruskal-Wallis test is the nonparametric equivalent of the analysis of variance and the Kruskal-Wallis test statistic has approximately a chi-squared distribution. Mapping of markers in linkage groups was performed with the JoinMap package (Stam, 1993).

Markers from map segments where genetic linkage with *Fusarium* resistance was suspected, were analysed as a backcross with interval mapping (Lander & Botstein, 1989), using MapQTL. This enabled the recovery of missing marker information with linked markers, and as such is more informative than one-way analysis of variance for a marker.

2. Results

2.1. Fusarium test

In the 1992 test 40 scale bulblets of 97 descendants and the three (grand)parents were tested for *Fusarium* resistance. Significant block effects and significant genotype effects were detected ($p < 0.001$). Block effects were due to three blocks, which were on one table. Those blocks had lower disease severity scores than the other seven blocks, probably because of stronger desiccation of the soil by mechanical ventilation. No correlation was found between the diameter of the scale bulblet planted and the disease rating score after harvest. The heritability of *Fusarium* resistance was estimated at 0.94 ($n = 40$). The cultivars Connecticut King (DSS = 1.86) and Pirate (DSS = 4.24, s.e.d. = 0.26 relative to 'Connecticut King') showed resistance and susceptibility, respectively as expected. The cultivar Orlito (DSS = 3.58, s.e.d. = 0.25), however, was found to be more susceptible than in earlier experiments (Straathof et al., 1993). The DSS values within the segregating population ranged from -0.34 to 4.54.

In 1993, 144 descendants and the three (grand)parents were tested. No block effects were found, the genotype effects were again highly significant ($p < 0.001$). No correlation between the size of the scale bulblets and the disease rating score was found. The heritability amounted 0.90 ($n = 20$). The cultivars Connecticut King and Orlito showed resistance (DSS = -0.19 and -0.18 (s.e.d. = 0.36), respectively), 'Pirate' showed susceptibility (DSS = 1.67, s.e.d. = 0.36). The DSS values within the population ranged from -1.21 to 1.78.

The correlation coefficient calculated between DSS values of the genotypes tested in 1992 and 1993 using data of 93 genotypes, including the (grand)parents was 0.61 (Figure 1). A distribution of the DSS values for the descendants tested in 1992 and 1993 is presented in Figure 2.

2.2. Morphological markers

Three morphological markers were scored on 98 descendants of the population during flowering in the greenhouse. Flower colours were scored as yellow or orange, flower spots were scored as present or absent and male sterility was scored as fertile or sterile. For flower colour and flower spots a 1:1 segregation and for male sterility a 3:1 segregation was found (Figure 3).

2.3. Association of RAPD markers with *Fusarium* resistance

213 segregating RAPD markers were tested for association with loci, involved in *Fusarium* resistance in either 1992 or 1993 using the Kruskal-Wallis test. All 150 descendants of the segregating population were used in this test for linkage, but the number of descendants evaluated per RAPD marker varied between 29 and 96 descendants for 1992 (average number of genotyped descendants per marker is 61) and between 29 and 128 for 1993 (average number of genotyped descendants per marker is 64). The Kruskal-Wallis test resulted in 24 significantly ($p < 0.1$) associated markers in 1992 and 36 markers in 1993, with 12 markers significant in both years (Table 1). Only three markers OPQ-08-05, OPS-13-03 and OPV-02-01 were significantly linked to the 1993 resistance data with $p < 0.005$.

2.4. Integral mapping results

The 213 RAPD markers, together with three morphological markers, were analysed with JoinMap in order to create a genetic map of this *Lilium* cross. With a significance

threshold for linkage of 3.0 LOD (^{10}log of odds) we were unable to separate the markers into a lower number of linkage groups. Even with a threshold of 2.5 LOD, more than 100 linkage groups were obtained.

Linkage of the dominant RAPD markers is hampered since they may segregate in the gametes of one parent of the cross, in the other, or in both (Figure 4A1-3, respectively). Markers segregating in one parent can be linked to each other (Figure 4B1). A marker segregating in one parent, however, cannot be linked directly to a marker segregating in the other parent (Figure 4B2). Markers segregating in one or the other parent can only be linked through the markers segregating in both parents (not shown).

Using a significance threshold of 2.5 LOD, linkage analysis of separate datasets for the 62 markers segregating in 'Connecticut King' yielded 45 linkage groups and for the 60 markers segregating in 'Orlito' 50 linkage groups were obtained.

Flower colour was linked to two RAPD markers (> 2.5 LOD). The morphological markers flower spots and male fertility are linked. Only 4 out of 98 descendants, which were scored for both traits were both sterile and had flower spots. Male fertility was also linked to two RAPD markers.

2.5. Maps of regions involved in *Fusarium* resistance

The linkage of the three RAPD markers highly associated with *Fusarium* resistance (OPQ-08-05, OPS-13-3, OPV-02-01) with other markers was further studied. When using a significance threshold of 2.5 LOD, OPS-13-3 could not be linked to any other RAPD marker. This marker segregates in the gametes of both parents (segregation type Figure 4A3), and the recessive allele is associated with a higher level of resistance. The markers OPQ-08-05 and OPV-02-01 formed a linkage group with four or three other markers, respectively (Figure 5). For both groups, Kruskal-Wallis test statistics are also presented in Figure 5. For OPQ-08-05 the recessive allele, whereas for OPV-02-01 the dominant allele is associated with a higher level of resistance. Both markers segregate in the gametes originating from 'Connecticut King' (segregation type Figure 4A1).

Markers of both map segments with the same segregation type, were analysed with interval mapping. The resulting estimates of phenotypic variance explained by the markers in the 1993 experiment were 7% for OPQ-08-05, and 8 % for OPV-02-01. The estimation of the phenotypic variance explained by marker OPS13-03 by one-way analysis of variance was 9%. This amounts to approximately 24% of the total phenotypic variance explained by the three markers together.

3. Discussion

In this initial study to link *Fusarium* resistance in lily to RAPD markers, three significant ($p < 0.005$) markers are found linked to resistance as determined in the 1993 test. Only one of these loci, however, was linked with the same significance with the results of the 1992 *Fusarium* test. Furthermore, both tests were not highly correlated and the resistant cultivar Orlito was scored susceptible in the 1992 test. Although clonal tests for *Fusarium* resistance in lily have proven to be reliable (Straathof et al., 1993; Straathof & Löffler, 1994a), in this experiment the repeatability was insufficient. For the first time, *in vitro* propagated scale bulblets were tested in large numbers (1992 test). Although preliminary experiments showed an acceptable correlation (data not shown) between *Fusarium* resistance of *in vitro* and *in vivo* propagated scale bulblets, several conditions *in vitro* could influence the *Fusarium* resistance. Since the initial number of scales was low, several propagation steps had to be made to obtain enough bulblets *in vitro*. This resulted in an tissue culture of 10 months with bulblets of different ages. Genotypes might obtain a

different physiological status during this culture. It is known that the nitrogen supply during cultivation of lily bulbs influence the *Fusarium* sensitivity (Linderman, 1977). For all these reasons, the results of the 1993 test are expected to be more reliable. A *Fusarium* test in 1994 using *in vivo* obtained scale bulblets will be carried out to confirm this assumption.

Two hundred and thirteen RAPD markers were used to create a genetic map, which resulted in fragmentary maps only. These fragmentary maps could be due to the following four reasons. First, the dominant RAPD markers used in this study segregate in three different ways (Figure 4A). Markers segregating in one parent can be linked to those segregating in the other parent through the markers segregating in both. The dominant markers segregating in both parents, however, provided so little linkage information that they were unable to perform this function. Second, markers were scored on subsets of all descendants only, resulting in a low numbers of plants in pairwise comparisons between markers. Third, the influence of the lily physical genome size on the genetical genome size. From mapping results in other crops it is known, however, that there is no direct relation between the physical and the genetical genome size (Nodari et al., 1993). Four, technical misscorings due to imperfect RAPD reactions and/or unclear band patterns. Repeatability of RAPD patterns in different labs and under different conditions is not always reliable (Penner et al., 1993). Furthermore, RAPD bands are sometimes difficult to score, when intensity varies due to experimental conditions or due to the presence of other bands that have almost the same size. For application of RAPD markers in plant breeding, highly linked markers have to be transformed to sequence characterised amplified regions (SCARs) (Paran & Michelmore, 1991).

Although no genetic map of lily is available, the distribution of the markers over the genome is unknown, and not all markers were tested on all 150 plants, still three markers highly significantly linked to *Fusarium* resistance were detected with the Kruskal-Wallis test. Therefore, this approach still seems promising to obtain markers linked to *Fusarium* resistance loci, which have not been detected so far. The use of a saturated linkage map of this population, however, is imperative in order to establish the number of loci involved in resistance and to ensure that markers identified to be linked with the Kruskal-Wallis analysis, but which are from different segregation types are not linked. To establish a saturated linkage map of this population, we are in the process of scoring more RAPD markers on all descendants and developing other PCR based marker types which inherit co-dominantly. To obtain more markers linked to the three markers already identified as being linked to *Fusarium* resistance loci, we are currently performing the bulked segregant analysis (Michelmore et al., 1991).

Large variation in resistance was found between descendants of the population. This resulted, in combination with a relatively low variation within genotypes, in a high heritability value. The heritability in 1992 was higher than in 1993, because of the higher number of scale bulblets used. The inheritance of *Fusarium* resistance did not show a clear Mendelian segregation (Figure 2). This could be due to the ordinal measurement scale or the genetic background. Transformation to the quantitative disease severity score scale might reduce the measurement problem. This transformation, however, may work counteractive, when the underlying genetic distribution is not normally distributed. The limited number of descendants used in our experiments make conventional inheritance studies speculative, especially if several genes are involved as expected in this population (Straathof & Löffler, 1994b).

The distribution pattern of the resistance level of the descendants showed that most descendants were as resistant as or even more resistant than both parents (Figure 2). Resistance linked to the absence of marker OPS-13-03 may contribute to a level of resistance, which is higher than the level present in 'Connecticut King' or 'Orlito'. This

result promises further possibilities for resistance breeding upon combining of alleles.

Three morphological markers were used in this study. Two traits (male sterility and flower spots) were found to be highly correlated. The inheritance of these traits is more complicated than shown in this study. Modifying genes are involved in flower colour (light/dark colour), spotting (few/many spots) and male sterility (functional male sterility).

The implications for practical breeding for *Fusarium* resistance in lily, when sufficient linkage is detected, are enormous. Estimates of how much can be gained with marker aided seedling selection depends on the number of loci and on how much these loci contribute to *Fusarium* resistance. This study does not reach definite conclusions about the number of loci involved in *Fusarium* resistance, but so far 24% of the resistance could be explained by the three markers. We speculate that more loci are involved in the coding for *Fusarium* resistance. The costs of testing seedlings on *Fusarium* resistance using linked markers, are strongly dependent upon the number of genes and consequently the number of PCR reactions which have to be performed. If many loci (e.g. ten) are involved in the coding for *Fusarium* resistance, costs for analysis may increase rapidly (e.g. 2-5 fold), depending on the number of SCAR reactions which can be combined in one PCR reaction.

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Table 1 List of markers linked significantly to *Fusarium* data in either 1992 or 1993.

RAPD marker	1992	1993	RAPD marker	1992	1993	RAPD marker	1992	1993
EJO-03-01	-[z]	**	OPQ-08-08	-	*	OPV-16-01	-	*
EJO-08-01	-	**	OPQ-15-03	***	**	OPV-18-01	-	*
OPA-01-03	-	*	OPR-03-02	*	-	OPV-18-02	-	*
OPB-03-01	-	**	OPR-05-01	*	*	OPW-03-01	-	*
OPB-15-01	*	**	OPR-08-02	**	**	OPW-05-01	**	-
OPB-17-01	*	-	OPS-13-03	**	****	OPW-11-01	-	**
OPB-20-02	*	-	OPT-17-04	*	-	OPX-01-01	*	**
OPC-06-01	*	*	OPU-01-01	**	**	OPX-03-03	**	-
OPC-09-01	-	**	OPU-02-01	*	**	OPX-04-03	*	-
OPE-20-01	-	**	OPV-02-01	****	****	OPX-09-01	-	*
OPH-01-03	-	**	OPV-02-02	-	**	OPX-18-01	**	-
OPP-20-03	-	*	OPV-02-03	*	-	OPY-11-01	-	*
OPQ-08-02	*	*	OPV-08-01	-	***	OPZ-03-01	-	*
OPQ-08-03	*	-	OPV-10-01	**	-	OPZ-03-03	-	**
OPQ-08-05	*	****	OPV-12-02	-	**	OPAA-04-01	-	**
OPQ-08-06	**	-	OPV-15-01	-	*	OPAE-03-01	-	**

[z]-, *, **, ***, ****, Nonsignificant or significant at P = 0.1, 0,05, 0.01, or 0.005, respectively, by Kruskal-Wallis test. Marker codes are according to the original codes given by Operon technologies. The suffices give the order of segregating polymorphism scored with one primer according to fragment size. EJO-03 and EJO-08 are the primers 3 and 8 as described by Williams et al. (1990).

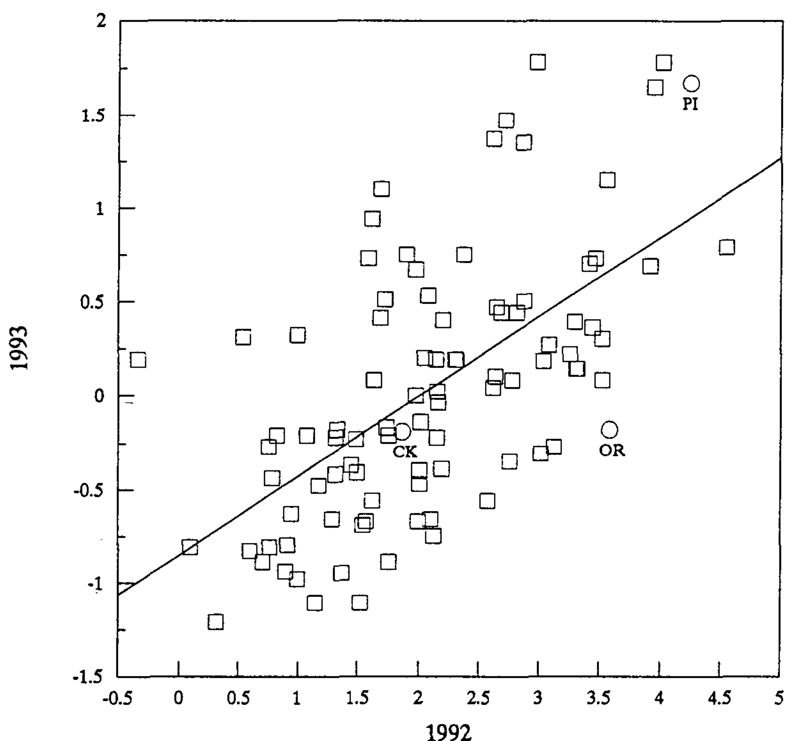

Figure 1 Correlation diagram between disease severity score values of the 1992 and 1993 *Fusarium* tests of 90 descendants of the lily cross 'Connecticut King' (CK) x 'Orlito' (OR), the two parents and the grandparent 'Pirate' (PI).

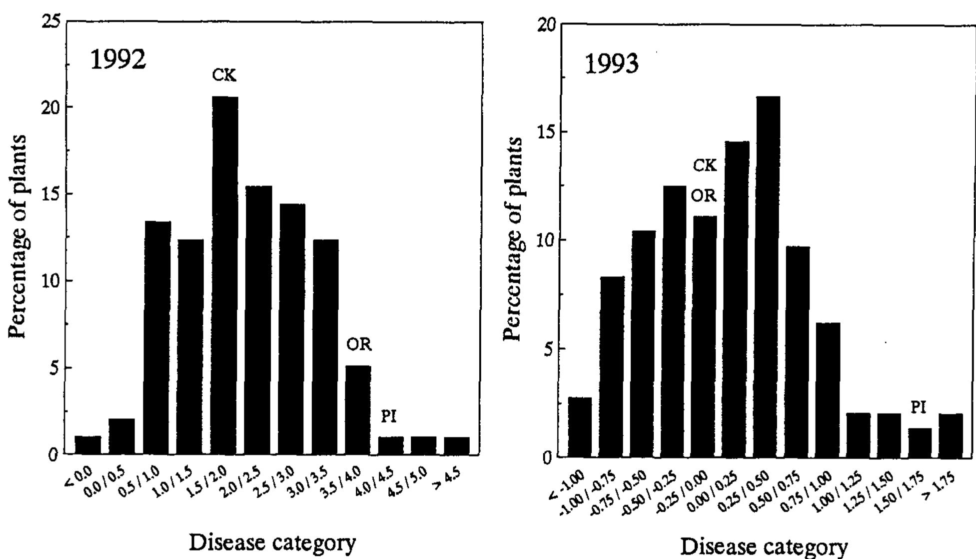

Figure 2 Distribution of disease severity score values in categories of 97 (1992) and 144 (1993) descendants of the lily cross 'Connecticut King' (CK) x 'Orlito' (OR) after screening for *Fusarium* resistance. The DSS values of the two parents and the grandparent 'Pirate' (PI) are indicated.

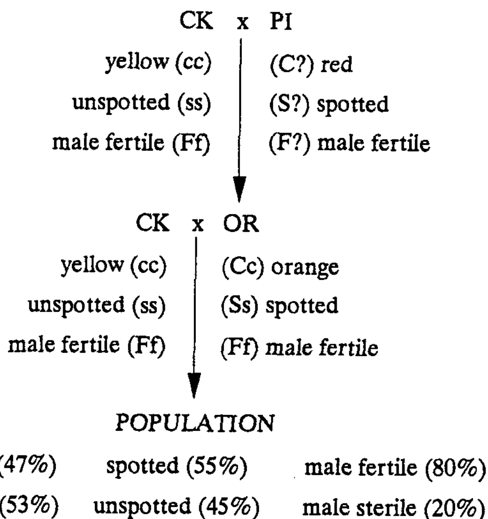

Figure 3 Genetic background and inheritance of flower colour (C), flower spots (S), and male fertility (F) in the crosses 'Connecticut King' (CK) x 'Pirate' (PI) and 'Connecticut King' (CK) x 'Orlito' (OR), percentages based on 98 descendants.

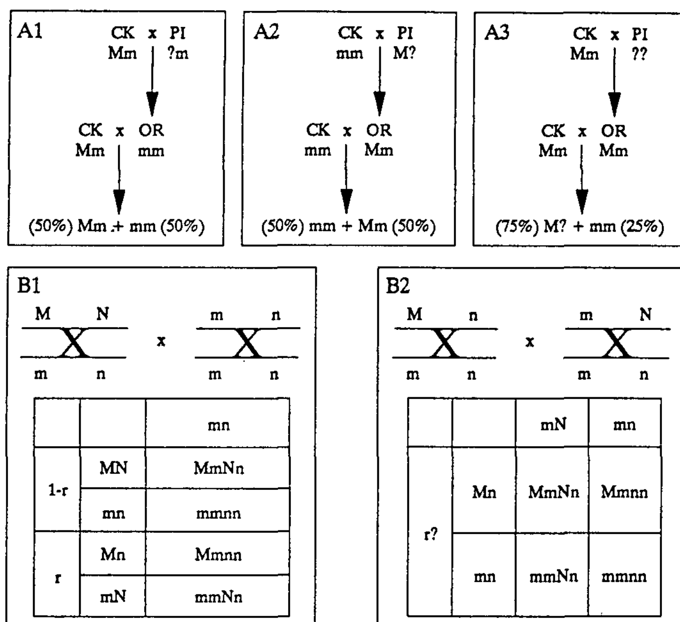

Figure 4 Three types of segregation of RAPD markers in the lily population 'Connecticut King' (CK) x 'Orlito' (OR = CK x 'Pirate' (PI)). Genotypes with the M. locus give a band, genotypes with the mm locus show no bands (A). If two markers (e.g. M and N) are derived from one parent, recombinant genotypes can be distinguished from nonrecombinant genotypes (B1). When markers are derived from two different parents, recombinants cannot be distinguished (B2). r = percentage of recombination.

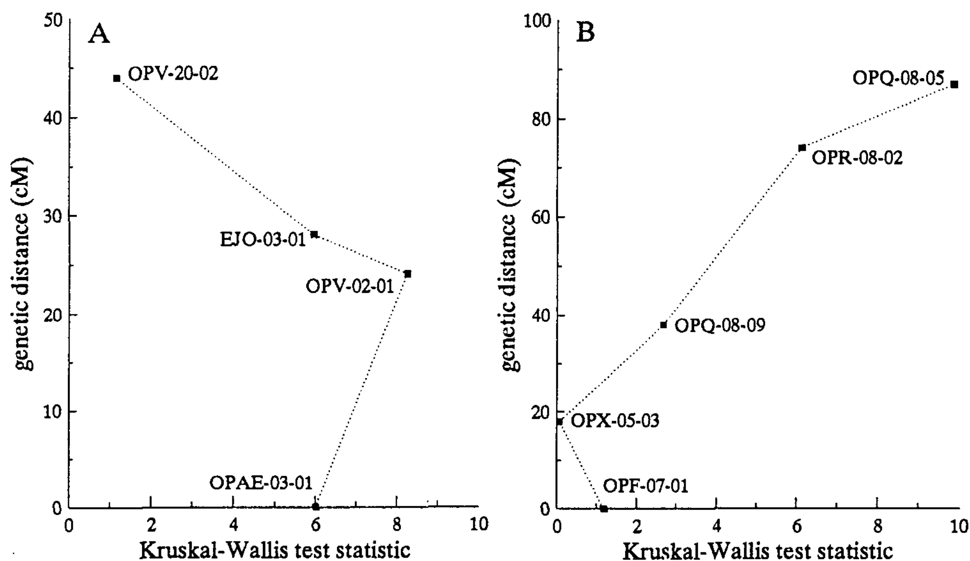

Figure 5 Maps of the two genomic regions, where *Fusarium* resistance loci were calculated to be linked to markers. A, the region including OPV-02-01 and B, the region including OPQ-08-05. The Kruskal-Wallis test statistic of 1993 of each marker is indicated right of the markers.