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Detection and analysis of QTLs based on RAPD markers for polygenic resistance to *Plasmodiophora brassicae* Woron in *Brassica oleracea* L.

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Abstract Resistance to *Plasmodiophora brassicae* Woron, the causal fungus of clubroot, was examined in an F_2 population of a cross between a clubroot-resistant kale (*Brassica oleracea* L. var. *acephala*) and a susceptible cauliflower (*Brassica oleracea* L. var. *botrytis*). QTL detection was performed with RAPD markers. Two resistance notations, carried out at different times after inoculation, were used. Three markers were associated with these two notations and three were specifically linked to only one notation. QTL analysis suggests the existence of at least two genetic mechanisms implicated in the resistance phenomenon.

Key words *Brassica oleracea* L. · *Plasmodiophora brassicae* Woron · RAPD markers · QTLs · disease resistance

Introduction

Clubroot, the most important root disease of cruciferous vegetable crops, is caused by *Plasmodiophora brassicae* Woron. This fungus induces severe worldwide crop losses to three important cultivated *Brassica* species : *B. oleracea* L., *B. napus* L. and *B. campestris* L. The disease is characterized by gall proliferation on the infected roots leading to problems in plant nutrition, to growth delay, and to an yield decrease.

Breeding of clubroot-resistant varieties is an important goal in cole crop improvement. Until now, no resistant cauliflower genotype has been identified. However, some sources of resistance to *P. brassicae* are known within the cabbage, kale and broccoli groups (Crete and Chiang 1980; Dixon and Robinson 1986; Crisp et al. 1989; Monteiro and Williams 1989; Dias et al. 1993). The resistance level of these sources is, however, variable and incomplete. In *Brassica oleracea* L. this character appears to be determined by several genes with either recessive (Weisaeth 1974; Crute et al. 1983) or dominant alleles (Laurens and Thomas 1993). Breeding for resistance is consequently difficult and no adequate results have been obtained for cauliflower. However, marker-aided selection can be very helpful, especially when the characters studied are polygenic and /or have a low heritability, a situation which is particularly common for resistance traits (Lefebvre and Chèvre 1995; Michelmore 1995). In the case of clubroot resistance management the detection of dominant QTLs (quantitative trait loci) could be of considerable value for breeding programs since their incorporation in susceptible genotypes would permit a direct increase of the resistance level in the improved genotypes.

Markers linked to QTLs implicated in the resistance to *P. brassicae* have been detected for *B.* oleracea by three research groups. Two QTLs for resistance to race 2 of *P. brassicae* were identified by Landry et al. (1992). In the case of a cabbage resistant line, alleles for resistance of two QTLs appeared to be dominant over susceptibility and the two QTLs accounted for 61% of the total variation for clubroot resistance. Figdore et al. (1993) have detected three QTLs in an F_2 population obtained from a cross between a *P. brassicae*-resistant broccoli and a *P. brassicae*-susceptible cauliflower. One dominant QTL came from the broccoli line, one QTL came from the cauliflower line and the third was due to a heterozygous state. These results indicated that it should be possible to select transgressive segregants particularly in F_1 hybrid genotypes. The authors suggested that the low chi-square values relative to a well-satured map indicate a tight linkage of the markers, with trait loci showing a minor effect. Using RAPD markers and BSA on doubled haploid lines, Pink et al. (1994) succeeded in selecting four RAPD bands associated with resistance and one with susceptibility. Three loci were linked to resistance to three collections of *P. brassicae* which differ in agressiveness. The other two loci were isolate-specific. The authors showed that several mechanisms, specific and non-specific, may be implicated in this host-parasite interaction.

Cole crops, especially cabbage and cauliflower, are economically important in Brittany where roughly 75% of the fields are infested with *P. brassicae* (Rouxel et al. 1991). Because of a lack of cultural methods, a resistance breeding program has been performed for cauliflower. Our laboratory owns an interesting kale line which

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presents a high level of resistance and a genetic study of the inheritance of resistance has been analyzed for the first time (Grandclément and Thomas 1996). The resistant character has been shown to be polygenic with a major dominance effect but low heritability. The present paper reports the detection, via RAPD markers, of QTLs conferring clubroot resistance, and consequently demonstrates two genetic mechanisms for resistance *P. brassicae* in.

Materials and methods

Plant material

The population studied was obtained from a cross between two *B. oleracea* lines: line C10, a resistant kale line used as pollinator, line 48.4.7, a susceptible cauliflower line employed as the female parent. From a single self-pollinated F_1 plant, 90 F_2 plants taken at random were analyzed. Seeds were sown on perlite in 12-cm-diameter dishes with lids. The resultant seedlings were transferred to pots (8 cm diameter) containing sterilized compost and grown in the greenhouse with five plants per pot.

Resistance testing

A field isolate of *P. brassicae,* used as the basic inoculum, was obtained from a naturally infested chinese cabbage crop. The plot was situated in an INRA station from western France. This isolate was characterized through the ECD set (European Clubroot Differential; Buczacki et al. 1975) as 16/31/31. Clubs were harvested, washed and stored at −18 °C.

Inoculum was prepared from slowly thawed out clubs. The clubs were ground in distilled water and filtered through three sieves (500 µ, 250 µ, 100 µ pore diameter). The concentration was adjusted with water to 10^6 spores/ml using a Malassez cell. The inoculation method chosen was the located watering method: 1 ml of inoculum suspension was placed at the bottom of the stem base of each seedling. The plants were allowed to grow during the summer months in a glasshouse under a maximum temperature of 20 °C.

Plants were first evaluated for clubroot 6 weeks after inoculation. Plants roots were thoroughly washed and records were made using the Buczacki et al. (1975) scale with one supplementary quotation (2^+):

0: no visible swelling
1: very slight swelling usually confined to lateral roots
2: moderate swelling on lateral roots and taproot
2^+: severe clubs on all roots but some roots remain
3: no root left, only one big gall

This record was used as a first resistance variable (R6W). Resistant plants (class 0) were replaced in individual pots with sterilized compost. Roots of the other plants were removed and the plants were re-grown in water so as to produce new roots. When new roots reached 3 cm, the young plants were replaced in individual pots with sterilized compost. For the parents, F_1, and F_2 populations, a disease index from 0 (no symptoms) to 100 (completely susceptible) was calculated by a summation of the coefficients (0, 25, 50, 75, 100) affecting each plant class frequency (Buczacki et al. 1975 modified by Laurens 1991).

Symptom development was regularly observed for 5–20% of the plants from class 0 and 1 so as to define the resistance phases. Two years after this first disease evaluation, a new assessment was performed using the same scale and pathological index. The results obtained were used as a second disease variable (R2Y)

QTL mapping

DNA extraction followed the CTAB method (Doyle and Doyle 1990) with a phenol chloroform step. The random sequence 10-mer primers used for the polymerase chain reaction (PCR) were obtained from Operon Technologies Inc. (Alameda, Calif.) Each PCR reaction was performed in a total volume of 10 µl containing 0.4 *Taq* polymerase (Eurobio) units, 150 µM of each dNTP, 0.2 µM of primer, 1.9 mM of $MgCl_2$, the amplification buffer from Eurobio, and 10–15 ng of genomic DNA. Samples for amplification were subjected to 50 cycles with three steps: 30 s at 92 °C, 1 min at 35 °C and 2 min at 72 °C, followed by a final extension of 5 min at 72 °C. DNA fragments were separated on 1.8% agarose gels, stained with ethidium bromide and then visualized using ultraviolet light.

Statistical analysis

The association of marker genotypes with clubroot resistance was assessed with one way ANOVAs at a significance level of $P < 0.05$. Using the F_2 population and RAPD markers, only two different genotypes were distinguishable; consequently, Student's *t*-test was chosen instead of the Fisher test. As the data were not normally distributed, even after transformation, the Kruskal and Wallis non-parametric test was also used. The tests were applied to two sets of plants: one with all individual F_2 plants, the other containing only those individual plants that were not included in class 0. The aim of this second set was to eliminate major effects and to make possible the detection of QTLs with minor effects.

Results

The 48.4.7 cauliflower parent line was scored as very susceptible, with plants belonging to classes 2^+ and 3 (disease index: 92.5%). The C10 kale parent line was scored as very resistant with all plants belonging to class 0 (disease index: 0). The disease mean score of the F_1 population (40 plants) was close to the C10 value (disease index: 3.2%) with most of the plants in class 0 and some in class 1. The disease index for the F_2 population was 38% for the R6W variable and 44% for the R2Y variable. F_2 plants were present in every class but with a higher number in extreme classes (Fig. 1.). For both variables, F_2 plant distributions did not correspond to normal distributions.

Fig. 1 Resistance to clubroot in *B. oleracea* measured in an F_2 population from the C10 × 48.4.7 cross. **a** Assessment obtained 6 weeks after inoculation. **b** Assessment obtained 2 years after inoculation

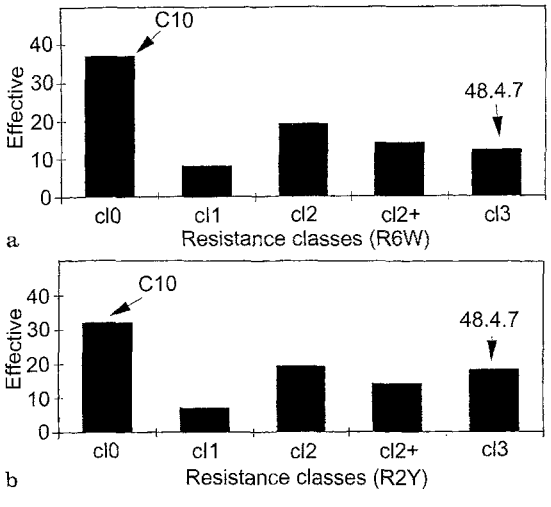

Table 1 RAPD markers linked to clubroot resistance variables in two F_2 sets of plants in *B. oleracea* L.

RAPD markers	Student's test value			
	Set 1-whole population		Set 2-population without plants from class 0	
	R6W[a]	R2Y[b]	R6W[a]	R2Y[b]
OPA4-700		2.26*		
OPA16-510		1.96*	2.55**	
OPB11-740			2.68**	2.30*
OPA1-1880			3.01**	2.08
OPL6-780			2.22*	
OPA18-1490			2.36*	
OPE20-1250				2.02*

* Significant at the 0.05 probability level; **: significant at the 0.01 probability level
[a] R6W: resistance assessment 6 weeks after inoculation
[b] R2Y: resistance assessment 2 years after the previous assessment (R6W)

For the whole F_2 population (first set), two markers had a significant *t*-test for clubroot resistance measured with the R2Y variable, but no marker was associated with the R6W variable (Table 1). The analysis of the second set (without plants from class 0) showed three markers linked to the resistance trait estimated with the R2Y variable, and six markers with the R6W variable (Table 1).

For both two variables, the same markers were detected with Student's *t*-test and the Kruskal and Wallis test.

Discussion

Resistance assessment shows a very high resistance level to *P. brassicae* W. in the kale parent C10. Moreover, this trait seems to be dominant in the F_1 and easily inherited in the F_2 population.

The similarity of the results obtained with Student's test and with the Kruskal and Wallis test proves the robustness of the variance analysis, even for variables with a non-normal distribution.

A non-normal distribution in many resistant plants may indicate the existence of a major dominant resistance gene. In order to detect minor effects, an analysis of less resistant individuals was carried out by Adam-Blondon et al. (1994). They showed that a higher number of markers were then associated with resistance variables. In our study, no marker was linked to the R6W variable in the whole population, whereas six markers were associated with it when the less resistant sub-population was analyzed.

A genetic map has been initiated from the same cross (Grandclément 1995) and four markers linked to resistance (OPL6-780, OPB11-740, OPA18-1490, OPA4-700) have been located on three linkage groups. Markers OPL6-780 and OPB11-740 were present on the same linkage group, separated by a distance of 15 cM. An important interval was influential on the trait. We observed either a wide QTL with many minor linked genes, or else a major QTL located in the middle of the two markers. Three other markers (OPE20-1250, OPA1-1880 and OPA16-510) were not associated with any of the previous linkage groups.

The use of two resistance variables may allow a better understanding of resistance mechanisms. The detection of different markers linked to the two resistance variables may indicate the influence of several different mechanisms during infection by *P. brassicae*. The life cycle of most pathogens requires distinct phases of interaction with its host and different genetic interactions may occur between the plant and the pathogen during each stage of infection (Michelmore 1995). Consequently an analysis of interaction at each stage may reveal the genes controlling each infection phase.

Thus, the cycle of *P. brassicae* is composed of two phases, one occurring in the root hair, and one in the living cortex and stele cells of the root. After an infection of root hairs by primary zoospores, plasmodia and zoosporangia are formed. This step is followed by the production of secondary zoospores which are released into the soil. These zoospores either reinfect root hairs or fuse in pairs and infect root cortical cells leading to the hypertrophy of the host tissue and gall development (Ingram and Tommerup 1972; Buczacki 1983).

According to the *P. brassicae* cycle, resistance scored 6 weeks after inoculation (variable R6W) may correspond to an important curtailment of the first infection stages, presumably resulting from a limitation of hairy root infection (primary zoospore penetration, primary plasmodia development or maturation. Such events would partially prevent secondary zoospore formation, though these structures are essential to the following stage of infection and the appearance of symptoms. In plants with such a resistance, symptoms induced by secondary zoospore multiplication would not be observable 6 weeks after inoculation. A second reduction mechanism in infection would then occur, totally blocking secondary zoospore penetration or multiplication. Six-week-resistant plants with no second genetic resistance system will reach the same susceptibility class as totally susceptible genotypes but with an important delay. Indeed, the final resistance level bears no relation with zoospore number (Naiki et al. 1978). Several studies confirm the existence of these two resistance mechanisms. Partial resistance to hairy root infection is not correlated with final symptoms, indicating another later

resistance system in *Brassica oleracea* (Voorrips 1992). Williams et al. (1971), Butcher et al. (1974) and Dekhuijzen (1979) found the root hair stages of the life cycle of *P. brassicae* in resistant varieties. This hypothesis can explain the specific QTL detected for the R2Y variable. Resistance based on R6W would then correspond to a general mechanism such as the thickening of cell walls and would involve different races of *P. brassicae* or different soil pathogens. Such a phenomenon was also noted by Young et al. (1993) for mildew resistance in *Vigna radiata*. Plants had a first resistance locus mapped 65 days after inoculation and another QTL mapped 85 days after inoculation (QTL85). The authors suggest that QTL85 would correspond to a later system leading to an inhibition in the development of the fungus.

Although C10 kale resistance appears to be dominant and heritable, its incorporation into cauliflower genotypes by traditional back-cross methods is impossible. Indeed, the BC_1 population has a disease index of 60% with 15% of the plants in class 0. For the BC_2 population, the disease index is 80.6% and no plant is observed in class 0. The identification of molecular markers tightly linked to the resistance QTLs will facilitate marker-assisted incorporation of the resistance QTLs detected. However, Figdore et al. (1993) found markers associated with both clubroot resistance and negative morphological traits (head color, head type). Pleiotropy or linkage between such loci may explain the difficulties encountered in incorporating resistance into the desired morphotype. The use of both resistance and morphological markers seems then to be most judicious. This method was developed by Pink et al. (1994) with regenerated doubled haploid plants screened both for resistance and morphological markers during the tissue culture stage. Only those with the required genotype were weaned and seeded. This method can help in the recovery of clubroot-resistant segregants with adequate cauliflower curd morphology. However, as the criteria for cauliflower marketing are very strict, all traits cannot be mapped. Morever, the use of many markers also rapidly increases the selection cost. A combination of molecular and phenotypic assessment is thus suggested in order to permit the early development of clubroot resistance in cauliflower.

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