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# Identification of RAPD markers linked to a *Phytophthora fragariae* resistance gene (*Rpf1*) in the cultivated strawberry

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**Abstract** Bulked segregant analysis (BSA) was used to identify seven random amplified polymorphic DNA (RAPD) markers linked to the *Rpf1* gene. *Rpf1* confers resistance to *Phytophthora fragariae* var. *fragariae*, the causal agent of red stele root rot in *Fragaria* spp. The bulked DNAs represented subsets of a $F_1$ population obtained from the cross Md683 × Senga Sengana which consisted of 60 plants and segregated in a 1:1 ratio for resistance or susceptibility to race 2.3.4 isolate NS2 of *P. fragariae*. Seven markers were shown to be linked to *Rpf1* and were generated from four primers; five of these markers were in coupling phase and two in repulsion phase with respect to the gene. A linkage map of this resistance gene region was generated using JoinMap 2.0™. The manner in which *Rpf1* and the linked markers co-segregated indicated that they are inherited in a disomic fashion. These markers could enable gene pyramiding and marker-assisted selection of resistance genes in strawberry breeding programmes.

**Key words** Red stele · Linkage-analysis · *Fragaria* × *ananassa* · Bulked-segregant-analysis (BSA) · Resistance-gene mapping

## Introduction

The cultivation of strawberries (*Fragaria* × *ananassa*) is limited by several diseases, including red stele (red core) root rot caused by the fungus *Phytophthora fragariae* var. *fragariae* (Hickman 1940). Symptoms of this disease are dwarfism, wilting, and reddening of the stele. Chemical treatment such as fumigation with methyl bromide and chloropicrin helps to reduce the inoculum potential in the soil but is hazardous to the environment.

Resistance to *P. fragariae* has long been assumed to be polygenically inherited (Stembridge and Scott 1959; Scott et al. 1984) but Van de Weg (1989, 1997) found evidence that red stele resistance in strawberry and the corresponding avirulence in *P. fragariae* interact according to a gene-for-gene system. At least five race-specific plant resistance genes and corresponding avirulence genes are believed to exist (Van de Weg 1997). The dominant *Rpf1* resistance gene was shown to segregate monogenically (Van de Weg et al. 1997).

One of the main goals of crop-breeding programmes is to combine excellent horticultural characteristics with high levels of resistance to the various pathogens. Genes conferring resistance to different races of *P. fragariae* are known to be present in some modern strawberry cultivars (Maas et al. 1989; Nickerson and Maas 1991; Kennedy and Duncan 1993; Van de Weg 1997). *Rpf1* confers resistance to at least 16 races, including the American isolates A1, A2, A3, A4, A6, A9, A10 and the Nova Scotian isolates NS1 and NS2 (Nickerson and Murray 1993; Van de Weg 1997). In the breeding of strawberry, epistatic interactions among resistance genes creates problems when screening is done by classical methods for multiple gene resistance (Van de Weg 1989). This reduces the efficiency of disease tests and slows down the development of new cultivars. This problem can possibly be solved by indirect selection using molecular markers linked to individual resistance genes.

Genetic maps defining linkages between molecular markers and genes of interest are useful tools for plant breeders. Marker-assisted selection has been shown to be a viable method for the improvement of disease and insect-pest resistance. In this context, different types of

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molecular markers have been employed, such as random amplified polymorphic markers (RAPD) and restriction fragment length polymorphism (RFLP) markers. RAPD markers (Welsh and McClelland 1990; Williams et al. 1990) are particularly easily and economically assayed, and they segregate in Mendelian fashion. They have been used in various crop species to map and tag resistance genes (Barua et al. 1993; Paran and Michelmore 1993; Chunwongse et al. 1994; Van der Beek et al. 1994; Poulson et al. 1995).

Mapping in the cultivated strawberry is complicated by its octoploid ($2n = 8x = 56$) genome structure. However, when a trait is controlled by a single dominant gene that segregates in a disomic fashion, it is possible to identify linked molecular markers. Bulked segregant analysis (BSA) (Michelmore et al. 1991) is the most efficient method of identifying markers linked to single genes of interest. This approach was used to tag disease-resistance genes in tomato (Van der Beek et al. 1994), common bean (Haley et al. 1993), lettuce (Paran and Michelmore 1993), potato (Pineda et al. 1993) and barley (Poulson et al. 1995). In the present report, application of BSA was successfully applied to screen a strawberry population segregating 1:1 for the *Rpf1* gene, and RAPD markers closely linked to and flanking this resistance gene were identified.

## Materials and methods

### Plant material

From the cross of two strawberry genotypes, Md683 (*Rpf1*; resistant) and Senga Sengana (*rpf1*; susceptible), 63 $F_1$ progeny plants were obtained. The $F_1$ plants were grown and runner-propagated in a greenhouse and previously characterised as resistant or susceptible to race 2.3.4 isolate NS2-25 (Nickerson and Murray 1993) of *P. fragariae* as reported by Van de Weg et al. (1997). Two severely diseased and one symptomless plant died prior to molecular analysis. The distribution of the average disease rating among the remaining 60 plants is summarised in Table 1. All plant material was developed and maintained at CPRO-DLO, The Netherlands.

### DNA isolation

DNA was isolated using a modification of the method of Torres et al. (1993). Three to five young (partially expanded) leaves per plant (0.08–1.0 g fresh weight) were ground to a fine powder in liquid nitrogen using a mortar and pestle. In the mortar, 1 ml of a 2% CTAB buffer, (100 mM Tris-HCl, pH 8.0; 1.4 M NaCl; 20 mM EDTA; 2% Hexa-decyl-tri-methyl-ammonium bromide, Sigma Chemical Co., Mo.) and 0.4% $\beta$-mercaptoethanol was added just before use, mixed to a slurry, and then transferred to a 1.5 ml microfuge tube containing 100 $\mu$l of chloroform/isoamylalcohol (24:1). Samples were vortexed briefly, incubated at 65°C for 45–60 min, and then cooled to room temperature. Samples were shaken vigorously to form an emulsion and then centrifuged at 14 000 g for 5 min. The aqueous phase was transferred to a new 1.5-ml Eppendorf tube and the DNA was precipitated by the addition of 1.0 ml of ice-cold 95% ethanol. Sample tubes were gently

**Table 1** Frequency distribution of average disease ratings for *P. fragariae* of 60 $F_1$ progeny plants from the cross Md683 × Senga Sengana. Disease severity was scored on a scale of 0 to 6; a 0 indicated no infection while a 6 indicated 75–100% infection of the roots. Averages are the mean of four replicate scores. This table is adapted from Van de Weg et al. (1997)

| Average disease index | % Root infection | Number of plants |
|---|---|---|
| 0 | 0 | 5 |
| 0–1 | $4 > RI > 0$ | 12 |
| 1–2 | $10 > RI > 4$ | 12 |
| 2–3 | $25 > RI > 10$ | 0 |
| 3–4 | $50 > RI > 25$ | 1 |
| 4–5 | $75 > RI > 50$ | 3 |
| 5–6 | $100 > RI > 75$ | 27 |
| Total | | 60 |

inverted and placed on ice for 10 min, then centrifuged at 14 000 g for 5 min. The pelleted DNA was washed in 70% ethanol, dried, and re-suspended in 25 µl of TE, pH 8.0. To each sample, 10 mg/ml of RNAse A (5 prime → 3 prime Inc., Boulder, COL) was added and heated to 37°C for 60 min. DNA was quantified and diluted to a working solution of 50 ng/ml.

### PCR conditions

RAPD primers were obtained from Operon Technology (Alameda, Calif.; kits A–Z, AA, and AB; 20 primers per kit) and the University of British Columbia (Vancouver, Canada; numbers: 2, 76, 84, 88, 89 102–106, 126, 131, 190, 192, 196, 200). Each 25 µl amplification reaction contained 50 ng of genomic DNA as a template. Amplification reactions contained 2.5 µl of 10× buffer [100 mM Tris-HCl, pH 9.0 (25°C), 500 mM KCl, 15 mM $MgCl_2$, 0.1% (w/v) gelatin and 1% Triton X-100], 100 mM of each dNTP, 30 ng of primer and 0.25 U of Super*Taq* polymerase (SphaeroQ, Leiden, NL) overlaid with sterile mineral oil. Amplifications were performed in a Perkin Elmer Cetus DNA 480 Thermal Cycler or a Hybaid Thermal Cycler and programmed as follows: 50 s at 94°C, a slow 1.5-min ramp to 34°C for 2 min and a 45-s ramp to 72°C for 2 min for 40 cycles. The reaction was then held at 72°C for 7 min before cooling to 4°C or room temperature. Amplification products were resolved by electrophoresis in a 2.0% TBE agarose gel.

### Bulked segregant analysis

DNA of nine resistant (R) and ten susceptible (S) plants composed the respective R and S bulks. A total of 576 RAPD primers were tested on these bulks. Each primer that detected polymorphisms was used twice more, then tested on the 19 individuals comprising the bulks. Putatively linked markers were then tested on the entire mapping population of 60 plants. Markers with confirmed linkage to *Rpf1* were re-tested twice at the population level to confirm their reproducibility.

### Linkage map of the *Rpf1* region

Map positions of the RAPD markers and the *Rpf1* gene were calculated with JoinMap 2.0™ (Stam 1993; Stam and Van Ooijen 1995) with a minimum LOD of 3 using the Kosambi function.

## Results

### Screening of RAPD polymorphisms by BSA

From the 576 primers tested, 42 generated 52 putative polymorphisms. One primer detected three polymorphisms, four primers detected two, and 37 primers detected one polymorphism each. Most of these polymorphisms were stably reproducible. However, when the nine 'R' and ten 'S' plants of the bulks were tested as individual DNA samples, segregation within the 'R' and 'S' bulks was observed for 35 out of the 52 initially identified polymorphic bands. These 35 polymorphisms resulted from unequal segregation of unlinked markers, possibly due to sampling error. Segregation patterns of 2 : 7, 2 : 8, and 3 : 7 were common. The analysis was continued for the markers the bulks showed at most two recombinants. Seventeen polymorphisms produced from 14 primers were tested on the entire population. Seven of these were shown to be linked to *Rpf1* and were produced from four primers of which the sequences are indicated in Table 2. These seven markers were present in Md683 but not in Senga Sengana.

### Analysis of the RAPD markers

Five of the seven markers segregated in coupling phase to *Rpf1*, while two segregated in repulsion phase (Fig. 1). Primers OPC-08 and OPV-02 each generated one marker while primer OPO-08 yielded two markers to *Rpf1* (Fig. 1). The OPO-08 markers have a relatively high molecular weight (1650 bp for OPO-08A and 1800 bp for OPO-08F) and are difficult to score due to a bright monomorphic band of 1700 bp (data not shown). Primer OPO-16 detected three markers at the same position to *Rpf1*. Two of them, OPO-16A and OPO-16B, are in coupling phase to *Rpf1* and were faint bands. The third marker, OPO-16C, is in repulsion phase to *Rpf1* and was an exceptionally bright band (Fig. 2). Sequence analysis will indicate if the three OPO-16 markers are alternate alleles of the same locus or of two closely linked loci. The number of recombinants between the markers of Fig. 1 are, from top to bottom, 7, 2, 1, 2, 0, 0, and 4. These numbers include two double cross-overs; one between the OPO-16 markers and respectively *Rpf1* and OPV-02D, and the other between OPO-08F and respectively OPO-08A and OPC8D.

**Table 2** Nucleotide sequences of four primers amplifying RAPD markers for *Rpf1* in the $F_1$ progeny from the cross Md683 × Senga Sengana

| Primers | 5′ Sequence 3′ |
|---|---|
| OPC-08 | TGGACCGGTG |
| OPO-08 | CCTCCAGTGT |
| OPO-16 | TCGGCGGTTC |
| OPV-02 | AGTCACTCCC |

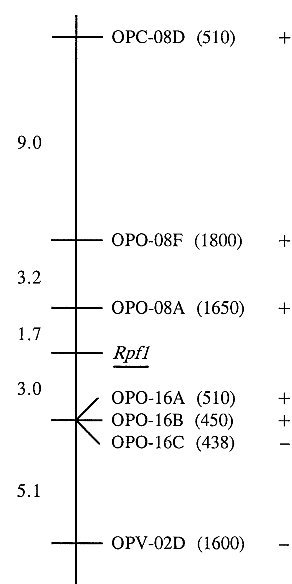


**Fig. 1** Linkage map of the region surrounding the *Rpf1* resistance gene and seven RAPD DNA markers, in the $F_1$ of the cross Md683 × Senga Sengana of the cultivated strawberry. Map distances, written on the left, are in centiMorgans. The fragment size of the marker is given in parentheses. The phase of each marker is indicated by either a (+), coupling phase, or by a (−), repulsion phase

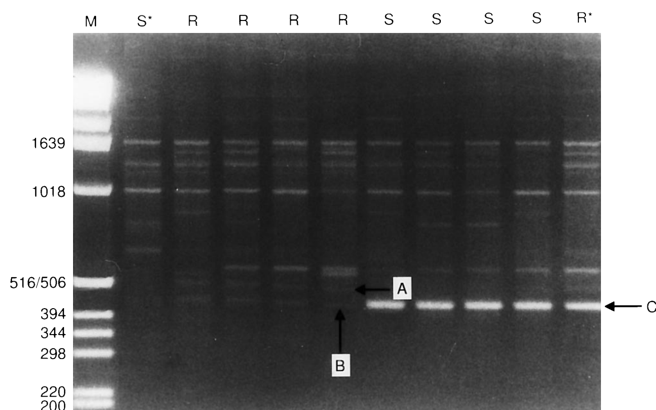


**Fig. 2** Individuals composing the resistant and susceptible bulks to *P. fragariae* isolate NS2-25 as screened with the RAPD primer OPO-16. Four resistant (*R*) and four susceptible (*S*) $F_1$ plants and the two parental genotypes were scored for the presence of the polymorphic markers denoted by the *arrows*. Parental plants are indicated by *R** (Md683) and *S** (Senga Sengana). Markers OPO-16A, B are in coupling phase while OPO-16C is in repulsion with the *Rpf1* resistance allele (Fig. 1)

## Discussion

The cluster of seven RAPD markers linked to *Rpf1* described here constitutes the first report of genetic linkage in the octoploid strawberry *F.* × *ananassa*. The mapping of the *Rpf1* locus was possible due to the disomic behaviour of the region and the reliable classification into resistant and susceptible individuals of the strawberry progeny. The results showed that BSA is an useful procedure in the strawberry to identify molecular markers for a single, dominant gene.

### Disomy

The data show that at least part of the genome of the octoploid (commercial) strawberry is diploid in nature. A 1 : 1 segregation in di- or poly somic inheritance can occur only in the case of a single locus when a simplex genotype is crossed with a nulliplex (recessive for all chromosomes). Disomy was demonstrated by the co-segregation of two such loci, *Rpf1* and the OPO-16C RAPD marker linked to the gene. For example, OPO-16C was present in 7% ($=2/29 \times 100\%$) of the resistant and in 100% of the susceptible progeny plants. These percentages can only be explained by disomic segregation of two linked loci at circa 3.0 cM but not by tetrasomic or octosomic inheritance. In a situation where the dominant alleles at two linked loci (e.g. *Rpf1* and the of OPO-16C marker) are not on the same chromosome, in repulsion, and under tetrasomic inheritance, then 33% of the resistant plants should have the marker present when the genes are absolutely linked. Under similar conditions with octosomic inheritance the marker would have been present in 43% of the resistant progeny. Disomy is further demonstrated by the co-segregation of OPO-16C and OPO-16A. Each descendent had only one of these markers, whereas with tetrasomic inheritance 16.6% would have had each marker, and 16.6% would have neither.

The data provide useful insight into the question of meiotic chromosome pairing patterns in the octoploid strawberry. Cytological data in *F.* × *ananassa* is controversial in that some studies showed primarily bivalent pairing during meiosis while others had mainly multivalent pairing (see review by Galletta and Maas 1990). Furthermore, the lack of morphological differentiation among strawberry chromosomes precludes the direct determination of whether bivalent chromosome pairing is fully preferential (autosyndetic) in the octoploid strawberry. In our results, the joint segregation pattern of OPO-16A and -16B versus OPO-16C can only be explained on the basis that each of the respective chromosomes are homologous and pair preferentially in Md683. If they did not pair preferentially, then some individuals in the progeny would be expected to carry either all three or none of these markers, as discussed above.

Formerly, part of the *F.* × *ananassa* genome was shown to behave as a diploid by the segregation patterns of phosphogluco isomerase isozymes (PGI) and leucine phospho isomerase (LAP) (Arulsekar et al. 1981; Kong and Sjulin 1993).

### Indirect selection

Md683 and its descendants have been used as founding sources of resistance in various breeding programmes. *Rpf1* is present in the cultivars created at the USDA, Beltsville, such as Allstar, Surecrop, and Lester, in Canadian cultivars such as Annapolis and Cornwallis, and in selections of the Scottish and the CPRO-DLO breeding programmes (Van de Weg 1997).

The establishment of markers linked to *Rpf1* confirmed the existence of this locus as a monogenic determinant of resistance to *P. fragariae*, thus supporting the proposed gene-for-gene model in the strawberry (Van de Weg 1997). Currently we are screening whether these markers are still present in modern *Rpf1* cultivars. If so, they can be utilised for indirect selection to replace, at least in part, resistance tests at the plant level, as well as in the molecular identification of strawberry cultivars (Gidoni et al. 1994; Hancock et al. 1994; Levi et al. 1994; Parent and Pagé 1995). The only other reports of linkage in strawberry deal with isozymes linked to red vs yellow fruit colour and to runnering vs non-runnering in the diploid *F. vesca* (Williamson et al. 1995; Yu and Davis 1995). Their use for indirect selection in the octoploid strawberry still needs to be determined.

Strawberry breeding for disease resistance to *Verticillium* or *Collectotrichum*, and other traits of agronomic interest, would benefit from the availability of molecular markers. As shown here, BSA is a useful procedure to identify such markers.

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