Development of SCAR Markers Linked to a *Phytophthora fragariae* Resistance Gene and Their Assessment in European and North American Strawberry Genotypes

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ABSTRACT. Two dominant sequence characterized amplified region (SCAR) markers (linked at 3.0 cM, coupling phase) were constructed for the strawberry (*Fragaria × ananassa* Duch.) gene *Rpfl*. This gene confers resistance to red stele root rot, caused by the soil-born fungus *Phytophthora fragariae* Hickman var. *fragariae*. The SCAR markers were developed originally from the sequence of RAPD OPO-16C₍₄₃₈₎ that is linked in repulsion phase to the *Rpfl* allele. This SCAR primer set produced multiple bands in the resistant test progeny and in some of the susceptible progeny; therefore, new SCARs were developed based on the sequence differences among these bands. These new SCARs were linked in coupling phase to the *Rpfl* allele and mapped to the same location as the original RAPD OPO-16C₍₄₃₈₎. The SCAR markers, as well as some additional RAPD markers known to be linked to *Rpfl*, were shown to be highly conserved in linkage to the gene based on examination of 133 European and North American *Fragaria* L. sp. cultivars and breeding selections. These flanking RAPD and SCAR-PCR markers can be used in breeding programs for the selection of red stele (*Rpfl*) resistance.

Many commercial strawberry (*Fragaria × ananassa*) cultivars are susceptible to red stele root rot caused by the soilborne fungus *Phytophthora fragariae* var. *fragariae* (Hickman, 1940). Symptoms of the disease can include dwarfism, wilting of leaves and petioles, reddening of the root stele, and eventual plant death. Soil disinfection with chemical fumigants such as methyl bromide or chloropicrin helps to reduce the inoculum potential of *P. fragariae* (Bollen, 1972). However, long-term application of the such fungicides as metalxy [N-(2,6-dimethylphenyl)-N-(methoxyacetyl)-DL-alanine methyl ester], and fosetyl-aluminum [aluminum tris (O-ethyl phosphonate)], can result in selection of resistant strains of the fungus (Seemüller and Sun, 1989). Therefore, development of resistant cultivars is highly desirable. The need for naturally resistant strawberry lines is desirable since one

of the main chemicals for control of *P. fragariae*, methyl bromide, is being phased out by the year 2006.

In strawberry, five genes for resistance to thirty races of *P. fragariae* have been described (Van de Weg, 1997); including *Rpfl*, a dominant gene (Van de Weg et al., 1997b) that confers resistance to at least 18 races of *P. fragariae* (Kennedy and Duncan, 1988; Nickerson and Murray, 1993; Scheewe, 1994; Van de Weg et al., 1997a). Previously, bulked segregant analysis (BSA) (Michelmore et al., 1991) was used to find eight randomly amplified polymorphic DNA (RAPD) markers linked to *Rpfl* (Haymes et al., 1997). Mapping this gene provided molecular support to its monogenic inheritance (Van de Weg et al., 1997b).

Molecular markers have many applications in plant breeding programs including marker-assisted selection, allowing breeders to maximize efficiency. One type of molecular marker, RAPD (Williams et al., 1990), has revolutionized genetic analysis and genome characterization for cultivated crops (for reviews see Devos and Gale, 1992; Tingey and Del Tufo, 1993; Waugh and Powell, 1992). More recently, RAPD markers linked to horticultural characteristics have been converted to specific and highly reproducible markers called sequence characterized amplified region (SCAR) markers (Paran and Michelmore, 1993).

In this paper, we report the cloning and sequencing of a RAPD marker linked to *Rpfl* and its conversion into two highly specific SCAR markers. Moreover, the maintenance of these SCARs and

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some RAPD markers in European and North American strawberry cultivars and breeding selections was examined.

Material and Methods

PLANT MATERIAL. A testcross between two *Fragaria xananassa* cultivars, Md683 (*Rpfl*, resistant) x Senga Sengana (*rpfl*,

Fig. 1. Sequence homology between the susceptible OPO-16C and the corresponding resistant band fragment of both the upper (U), middle (M), and lower (L) resistant band fragment amplified with the SCAR primers from Table 1 and Fig. 2. Alignment and homology analysis was done using multiple sequence alignment and cluster analysis. Base pair changes are indicated directly below the sequence, deletions are noted by the symbol (-). The symbol (*) indicates two Gs at position 270. The original RAPD primer is underlined.

susceptible), was performed. The resulting progeny of 60 F_1 plants segregated in a 1:1 ratio for the *Rpfl* locus (Van de Weg et al., 1997b), and were used for the initial mapping of this gene with RAPD markers (Haymes et al., 1997). The same population was used in the present study to develop the SCAR markers and test their segregation. For determination of marker presence in the present study, strawberry selections from the Centre for Plant Breeding and Reproduction Research, Department of Vegetable and Fruit Crops (CPRO-DLO), Wageningen, The Netherlands, strawberry collection and breeding program or from K. Hummer of the U.S. Department of Agriculture (USDA), National Germplasm Repository, Corvallis, Ore., were used. In total, 68 European and 65 North American cultivars and selections were tested.

RESISTANCE TESTS. Resistance tests were performed at either CPRO-DLO or at the USDA-Agricultural Research Service (ARS) Fruit Laboratory, Beltsville, Md. At CPRO-DLO, *Rpfl* genotypes were identified as such by their resistance to isolate NS2 and susceptibility to one of the isolates A7, A8, NS3, and NS4 (Van de Weg, 1997). Tests were performed according to Van de Weg et al. (1997b). For the F_1 mapping population, 29 individuals were shown previously to be resistant and 31 susceptible to virulent race 2.3.4 isolate NS2-25 (Haymes et al. 1997).

At the USDA-ARS Fruit Laboratory, *Rpfl* genotypes were identified by their resistance to isolate A3 or to a mixture of isolates A1, A2, A3, A4, and A6. Occasionally, strawberry selections were also tested with a series of individual inoculations with isolates A1, A2, A4, and A6. Susceptibility to these isolates indicates absence of *Rpfl*. Tests were performed according to Maas et al. (1989) and Scott et al. (1975).

DNA ISOLATION AND PCR AMPLIFICATION OF RAPD MARKERS AND ISOLATION OF THE RAPD MARKER. DNA extraction of the testcross population was done according to Haymes et al. (1997) and that of the European and North American selections, also following procedures of Haymes (1996). Amplifications with RAPD primers were performed according to Haymes et al. (1997). For isolation of the RAPD marker OPO-16C₍₄₃₈₎, DNA from susceptible plants was amplified with the primer OPO-16: 5'-TCGGCGGTTC-3' (Operon Tech, Alameda, Calif.), and separated on a 2% agarose gel (1× TBE). The polymerase chain reaction (PCR) fragment corresponding to OPO-16C₍₄₃₈₎ [linked in repulsion at 3.0 cM from *Rpfl*; (Haymes et al., 1997)] was cut out of the gel. The gel fragment was placed into 25 μ L of 0.5× TBE for 30 min to allow the DNA to diffuse into the buffer. RAPD-PCR re-amplification of the OPO-16C fragment was performed by using 5 μ L of the DNA TBE solution, in a total volume of 50 μ L, and set up as described previously. The re-amplified OPO-16C fragment was electrophoresed in a 2% TBE agarose gel and the corresponding band fragment was again excised from the gel and placed into a dialysis tube containing 500 μ L 0.5× TBE, and electrophoresed for 5 min. This solution was transferred into a 1.5 mL Eppendorf tube, extracted with an equal volume of phenol/chloroform (1:1, v/v), mixed, and centrifuged at 14,000 g_n for 5 min. The supernatant was transferred into a new 1.5 mL Eppendorf tube and 1 mL ethanol (EtOH)-acetate mix [96 mL EtOH, 4 mL 3 M NaAc (pH 5.2)] was added. The tube was inverted gently and left at room temperature (23 °C) for 3 min before being centrifuged at 14,000 g_n for 5 min, after which the supernatant was discarded. The DNA was washed in 70% EtOH, dried and resuspended in 30 μ L sterile H₂O before quantification. The DNA was quantified both spectrophotometrically and by running the samples on a 1% TBE agarose gel with DNA standards.

Fig. 2. DNA of three resistant (R) and three susceptible (S) plants amplified with SCAR primer sets. (A) SCAR-S, (B) SCAR-R1A, and (C) SCAR-R1B. Amplified products were analyzed by electrophoresis on 2% TBE agarose gel; lanes 1 to 3 are *RpfI* resistant plants and lanes 4 to 6 are *rpfI* susceptible plants to *P. fragariae* isolate NS2-25. Molecular weights in (bp) are indicated by M. In A, lane 3, the upper, middle, and the lower band fragments are shown. Primer sequences are listed in Table 1.

CLONING AND SEQUENCING OF A RAPD MARKER. The purified DNA fragment amplified with RAPD marker OPO-16C₍₄₃₈₎ was cloned into the *Hinc* II site of plasmid pBluescript SK+ (Stratagene, La Jolla, Calif.), and transformed into *E. coli* DH5 α strain according to Sambrook et al. (1989). Recombinant clones were screened for appropriately sized inserts. The selected recombinant clones were sequenced from both sides on an Applied Biosystems 373 Automated Sequencer (Applied Biosystems, Inc., Foster City, Calif.) using a *Taq* DyeDeoxy Terminator Cycle Sequencing kit [Applied Biosystems (AB)]. Template DNA preparations and sequence reaction mixtures were done according to AB recommended procedures.

PCR FOR SCAR-S PRIMERS. A pair of 24 base pair (bp) primers (SCAR-S) were designed based on the sequence of the cloned OPO-16C₍₄₃₈₎ fragment (Fig. 1). Each PCR tube contained 50 ng each of the forward and reverse SCAR-S primers, 0.1 mM of each of four dNTP, 20 ng genomic DNA, 2.5 μ L 10 $\times$ reaction buffer (Life Technologies, Gaithersburg, Md.), 0.75 μ L 50 mM MgCl₂ (Life Technologies), 0.5 unit of *Taq* polymerase (Life Technologies) and 18 μ L of sterilized H₂O, making a total volume of 25 μ L. PCR amplification was conducted in either a PE Cetus Thermal Cycler 480 (PE, Foster City, Calif.) or a Hybaid Omnigene Cycler (Hybaid Omnigene, Franklin, Mass.). PCR conditions for both machines were 94 $^{\circ}$ C for 3 min followed by 25 cycles of 94 $^{\circ}$ C for 30 s, 60 $^{\circ}$ C for 45 s, 72 $^{\circ}$ C for 1 min, and a 7-min extension at 72 $^{\circ}$ C. The completed reactions were held at 4 $^{\circ}$ C for the PE Cycler and 20 $^{\circ}$ C for the Hybaid Omnigene Cycler.

GEL ANALYSIS AND SEQUENCING OF SCAR PRODUCTS AND DEVELOPMENT OF RESISTANCE-LINKED SCARS. DNA of five susceptible and five resistant plants from the testcross progeny was amplified using the SCAR-S primers. Electrophoresis of the PCR products on a 3% TBE agarose gel revealed multiple bands in some of the susceptible progeny and in all of the resistant progeny (Fig. 2). The amplified fragments were excised from the gel and purified (as stated previ-

ously). Sequencing reactions were set up as stated previously, except that 250 ng of purified DNA was used, and that the SCAR-S primers (10 μ M; $\approx$ 50 ng) were used instead of the forward and reverse M13 primers. Two primer sets based on sequence differences between resistant and susceptible individuals were developed and designated as SCAR-R1 primer sets (Table 1).

PCR USING SCAR-R1 PRIMERS. Samples for PCR were set up as stated for the SCAR-S amplification. Samples were analyzed by electrophoresis on a 2% TBE agarose gel. The PCR buffers were optimized for each of the two SCAR-R1 primer sets and differed solely for their pH; SCAR-R1A: 10 $\times$ buffer (100 mM Tris-HCl pH 8.8; 35 mM MgCl₂, 750 mM KCl) and SCAR-R1B 10 $\times$ buffer (100 mM Tris-HCl pH 9.2; 35 mM MgCl₂, 750 mM KCl) (Schoettlin et al., 1993).

RPF1 ASSESSMENT IN SELECTIONS TESTED AT CPRO AND USDA-ARS. Assessments for presence of the *RpfI* resistance (Tables 2 and 3) gene were based on previous testing of selections to individual isolates of the fungus using the gene-for-gene model for the strawberry-*P. fragariae* pathosystem (Van de Weg, 1997). Data came from resistance tests (see below) or from earlier reports, although data were not available for a few selections. For some Dutch accessions, conclusions on their lacking of *RpfI* were based on their susceptibility to naturally infested fields in The Netherlands on which *RpfI* was effective (W.E. Van de Weg and B. Meulenbroek, personal communication). In the case of North American strawberry cultivars introduced before 1945, judgement of their lacking the *RpfI* gene was based on susceptibility on fields infested naturally with *P. fragariae* (Darrow, 1966). These cultivars were selected initially before breeding for red stele resistance.

MOLECULAR MARKERS USED IN THE CONSERVATION STUDY. The SCARs were mapped in this population using the program JoinMap ver 2.0 (Stam, 1993; Stam and Van Ooijen, 1995) with a minimum LOD 5 using the Kosambi function. Three RAPD markers (OPC-8D, OPO-8A, and OPO-16C, (Operon Technol-

Table 1. SCAR primers based on the sequence differences between the OPO-16C RAPD marker for susceptibility and the sequenced markers for resistance from the SCAR-S primers. Map position, band fragment, and expected band length from which primers were designed are indicated.

Primer	Sequence	Map position	Band fragment	Expected band length
SCAR-S	Forward: 5'-TGC ATC ATT AAT GTA GAA GTC TTT-3'	29-52	(OPO-16C)	404
	Reverse: 5'-GGT TTC CCA AAA GAT TAG TAG TTA-3'	433-410	(OPO-16C)	
SCAR-R1A	Forward: 5'-TGC ATC ATT AAT GTA GAA GTC TTT-3'	29-52	(OPO-16C)	285
	Reverse: 5'-TGA TGC GAC ATA CAA AAA TAT TAG-3'	320-297	(Resist M)	
SCAR-R1B	Forward: 5'-ATG ACC GAA TCA AAA TAT TCT -3'	271-298	(Resist M)	133
	Reverse: 5'-ACT AAC ACA GAC AAC CCA CCA -3'	410-390	(ResistM)	

ogy, Alamelda, Calif.) and one SCAR marker (SCAR-R1A) linked to *Rpfl* (Haymes et al., 1997) were used for the conservation analysis. Their relative positions and linkage phase to *Rpfl* are presented in Fig. 3.

Results

DEVELOPMENT OF SCAR PRIMERS BASED ON THE SUSCEPTIBILITY ALLELE. The DNA fragment representing RAPD OPO-16C⁽⁴³⁸⁾

Table 2. Thirty-four *Rpfl*-resistant European and North American strawberry cultivars and selections tested with two RAPD markers and one SCAR marker [(+) present, (–) absent] for resistance to *Phytophthora fragaria*. Marker linkages are illustrated in Fig. 2. Male parents are in bold.

Genotype	Country of origin ²	Parental plants	OPO-16C (susc) ^y	OPC-8D (resist) ^y	OPO-8A (resist)	SCAR-R1A	<i>Rpfl</i> (resist) ^x
Allstar	USA	US4419 (Redstar x Surecrop) x [(MDUS 1972 x Midland) x (Redstar x Md430)] x (NC-1768 x Surecrop) x MDUS 3184 (NC-1768 x Surecrop)	–	+	+	+	+ a
Annapolis	CAN	Micmac x Raritan x Earliglow	–	–	+	+	+ a
Arking	USA	Cardinal x ARK-5431 [MDUS 3082 (NC-1768 x Surecrop) x Delite]	–	–	+	+	+ b
Auchincruive-6	UK	Frith x Frith	–	+	+	+	+ c
Benton	USA	ORUS 2414 x Vale	–	+	+	–	+ d
Cornwallis	CAN	Earliglow x Kent	+	+	+	+	+ a
CPRO 77191	NL	Guardian x Sivetta	+	+	+	–	+ e
CPRO 88218	NL	Bogota x Scott	+	+	–	–	+ e
CPRO 88239	NL	Bogota x Yalova-4	–	–	+	+	+ e
CPRO 88246	NL	(Redchief x Sivetta) x Bogota x Yalova-4	–	–	+	+	+ e
CPRO 88275	NL	Holiday x (Induka x Sivetta) x Yalova-4	–	–	–	+	+ e
CPRO 88310	NL	(Sivetta x Holiday) x Korona x Scott	+	+	–	–	+ e
CPRO 88312	NL	(Sivetta x Holiday) x Korona x Scott	+	+	+	–	+ e
CPRO 89027	NL	(Tamella x Redgauntlet) x Md2700 (Pocohantas x Stelemaster) x Allstar	–	+	–	+	+ e
CPRO 90025	NL	Allstar x Korona	+	+	+	+	+ e
Darrow	USA	Md2713 (Redglow x Surecrop) x MDUS 2787 (Fairland x Midland) x (Midland x MD683)	+	+	+	+	+ a
Delite	USA	Albritton x MDUS 2650 (Blakemore x MD683) x Midland x Fairpeake x (Aberdeen x Redheart)	+	–	+	–	+ a
Earliglow	USA	Md2359 (Fairland x Midland) x Md2713	+	+	+	+	+ a
Guardian	USA	NC-1768 [Fairpeake x (Aberdeen x Redheart)] x Tennessee Beauty x Surecrop	+	+	+	+	+ a
Hood	USA	ORUS 2315 x Puget Beauty	+	+	+	+	+ a
Linn	USA	MDUS 3184 x ORUS 2414	–	–	+	+	+ a
MD683	USA	Scotland BK-46 (Frith selfed) x Fairfax	+	+	+	+	+ a
MDUS 3184	USA	NC-1768 x Surecrop	+	+	+	+	+ b
Perle de Prague	UK	Unknown x Unknown	+	–	–	–	+ a
Redchief	USA	NC-1768 x Surecrop	+	+	+	+	+ a
Scott	USA	Sunrise x Tioga	+	+	+	–	+ f
Siltez	USA	ORUS 2012 x ORUS 1816	–	+	+	+	+ a
Stelemaster	USA	Fairland x MD683	+	+	+	–	+ a
Sunrise	USA	US 4152 (Tennessee Shipper x Maytime) x Stelemaster	+	+	+	–	+ a
Surecrop	USA	Fairland x MDUS 1972 (Blakemore x MD683)	–	+	+	+	+ f
Tribute	USA	EB 18 (NC-1768 x Surecrop) x Cal 65.65-601 x MDUS 4258 (Redglow x Surecrop) x (Midland x Sunrise)	+	–	+	–	+ f
Tristar	USA	EB 18 x MDUS 4258	+	+	+	+	+ a
Yalova-4	TU	Cengelköy x Aliso	–	–	+	+	+ e
Yalova-15	TU	Cengelköy x Tiago	+	–	+	+	+ e

²Plant material was obtained from CPRO-DLO strawberry collection and breeding program, The Netherlands for all Dutch (NL) selections, MD683, 'Perle de Prague', and the two selections from Turkey (TU), and all others came from the USDA National Germplasm Repository, Corvallis, Ore.

^yAbbreviations: susc = susceptible and resist = resistant.

^xOrigin of the data on which the presence or absence of *Rpfl* was based: a = Van de Weg (1997), b = artificial inoculation with known races at USDA/ARS, Beltsville, Md., USA, c = Van de Weg et al. (1997a), d = tests on naturally infested fields on which *Rpfl* is effective, Van de Weg (1997b), e = artificial inoculation with known races at CPRO-DLO, The Netherlands, f = Maas et al. (1989), g = resistance or susceptibility is based on the pedigree of the parents, h = Darrow (1966), and/or selected during a time when genotypes were not bred for red stele resistance, I = Hickman (1940), j = Stembridge (1961), k = unpublished data of the USDA/ARS, Beltsville, Md., USA, l = tests on naturally infested fields on which *Rpfl* is effective, Van De Weg & Meulenbroek unpublished data, m = Law and Milholland (1992).

Table 3. Fifty-three *Rpfl* susceptible (susc) European and North American strawberry cultivars and selections tested with two RAPD markers and one SCAR marker (+ present; – absent) for resistance (resist) to *Phytophthora fragariae*. The markers are identical to those in Table 2. Male parents are in bold.

Genotype	Country of origin ^z	Parental plants	OPO-16C (susc) ^y	OPC-8D (resist) ^y	OPO-8A (resist)	SCAR-R1A	<i>Rpfl</i> (resist) ^x
52 AC 18	UK	Unknown x Unknown	–	–	–	–	– a
53 Q 13	UK	Auchincruive 11 x <i>Fragaria virginiana</i>-1	+	+	–	–	– c
Aberdeen	UK	Unknown x Unknown	+	–	–	–	– a
Avanta	NL	Induka x Sivetta x Karina x Precoce di Romagna	+	–	–	–	– d,g
Blakemore	USA	Missionary x Howard 17	+	–	–	–	– a
Bogota	NL	Zb.53-11 x Tago	+	–	–	–	– e
Brighton	USA	Tufts x Cal 65.65-601	+	–	–	–	– h
Cal 42.8-16	USA	Sierra x (Blakemore x Nich Ohmer) x (Royal Sovereign x Howard 17) x (Royal Sovereign x Howard 17)	–	–	–	–	– h
Cambridge Favourite	UK	Etterburgseedling x Avant Tout x Blakemore	+	–	–	–	– i
Cambridge Vigour	UK	US 3378 (Aberdeen x Fairfax) x Early Cambridge	–	+	–	+	– a
Cavalier	CAN	Valentine (Howard 17 x Vanguard) x Sparkle	+	–	–	–	– g,j
Chandler	USA	Douglas x Cal 72.361-105	–	–	–	–	– e
Climax	UK	TD-8 (CC-6 O.P) Frith O.P) x Aberdeen	+	–	–	–	– a
Columbia	USA	WSU 157 x WSU 175	+	–	–	–	– b
CPRO 87018	NL	Elsanta x Cambridge Favourite x (Sivetta x Precoce di Romagna)	+	–	–	–	– e
CPRO 89028	NL	(Tamella x Redgauntlet) x Md2700 x Allstar	+	+	–	–	– e
CPRO 90013	NL	Rapella x Cambridge Favourite x Elsanta	+	–	–	–	– d,e,g,i
CPRO 90017	NL	Rapella x Cambridge Favourite x Gelria	+	+	–	–	– d,g,i
Del Norte	USA	<i>F. chiloensis</i> (random selection) x <i>F. chiloensis</i> (random selection)	–	–	–	–	– a
Douglas	USA	Tufts x Cal 64.57-108	–	–	–	–	– k
Elvira	NL	Gorella x Vola	+	–	–	–	– e
Fairfax	USA	Ettersburg 450 x Howard 17 Royal Sovereign x Howard 17 Howard Supreme x Ettersburg 450	+	–	–	–	– j
Florida Belle	USA	Sequoia x Earlibelle	+	–	–	–	– b
Gorella	NL	Juspa x MDUS 3763 (Suwannee x Midland)	–	–	–	–	– e
Grenadier	CAN	Valentine x Fairfax	+	–	–	–	– g
Holiday	USA	Raritan x NY-844 (Redglow x Tennessee Shipper) x Redglow	+	–	–	–	– a/g
Howard 17	USA	Crescent x Howard 1	+	–	–	–	– j
Jerseybelle	USA	NJ 953 (Lupton x Aberdeen) x Fairfax x NJ 925 (Pathfinder x Fairfax)	+	–	–	–	– l
Jucunda	?	Unknown x Unknown	–	–	–	–	– h
Karola	NL	Gorella x Midway x Karina	+	–	–	–	– e
Kent	CAN	Frogmore Late Pine x Raritan	+	–	–	–	– a
Lambada	NL	Sivetta x Holiday x Karina x x Primella	+	–	–	–	– e
Lupton	USA	Joe x Gandy	–	–	–	–	– h
Lassen	USA	Blakemore x (Marshall x Fendalcinno) x Nich Ohmer x (Royal Sovereign x Howard 17) x (Marshall x Fendalcinno)	–	–	–	–	– h
Macherauch's Frühernte	DL	Geneva x Deutsch Evern	+	–	–	–	– h
Marmolada	IT	Gorella x Unknown	–	–	–	–	– e
Marshall UCM 3585	USA	Un known x Unknown	+	–	–	–	– h
Micmac	CAN	Tioga x K61-87 (Guardsman S_p)	–	–	–	–	– a
Midland	USA	Howard 17 x Redheart	–	–	–	–	– j
Midway	USA	Dixieland x Temple	+	–	–	–	– f,m
Mrak	USA	Cal 69.141-101 (Hecker) x Aiko	+	–	–	–	– e
Redcoat	CAN	Sparkle x Valentine (Howard 17 x Vanguard)	+	–	–	–	– a,g

Table 3. Continued.

Genotype	Country of origin ^z	Parental plants	OPO-16C (susc) ^y	OPC-8D (resist) ^y	OPO-8A (resist)	SCAR-R1A	<i>Rpfl</i> (resist) ^x
Redgauntlet	UK	NJ-1051 x Climax	+	—	—	—	— a
Redglow	USA	Fairland x Tennessee Shipper	—	+	—	—	— b/g
Royal Sovereign	UK	Nobel x King of the Earlyies	+	—	—	—	— h
Saladin	UK	Auchincruive 61 AP 60 x MDUS 2650 (Blakemore x MD683) x Midland) x (Fairpeake x (Aberdeen x Redheart)	+	—	—	—	— a
Selva	USA	Cal 70.3-117 x Cal 71.98-605	—	—	—	—	— e
Senga Sengana	DL	Markee x Sieger	—	—	—	—	— a
Shucksan	USA	Northwest x Sierra x Columbia	+	—	—	—	— b
Sierra	USA	Nich Ohmer x (Royal Sovereign x Howard 17) x (Marshall x Howard 17)	+	—	—	—	— h
Talisman	UK	NJ-1051 x Climax	+	—	—	—	— e
Yaquina A	USA	<i>F. chiloensis</i> (random selection) x Unknown	—	—	—	—	— a
Yaquina B	USA	<i>F. chiloensis</i> (random selection) x Unknown	—	—	—	—	— a

^zPlant material was obtained from CPRO-DLO strawberry collection and breeding program, The Netherlands for all selections from Germany (DL), Italy (IT), The Netherlands (NL), United Kingdom (UK), except as noted below, Canadian (CAN) selections of 'Kent' and 'Micmac', and the USA selections, 'Blakemore', 'Del Norte', 'Holiday', 'Mrak', 'Selva', Yaquina A and Yaquina B and 'Jucunda' of unknown country origin. The other selections from the USA and Canada (CAN) came from the USDA National Germplasm Repository, Corvallis, Ore., as did the UK selections 'Cambridge Favourite', 'Cambridge Vigour', and 'Royal Sovereign'.

^yAbbreviations: susc = susceptible and resist = resistant.

^xRefer to footnote of Table 2.

was cloned and sequenced (Fig. 1). Using this sequence, a pair of SCAR-S primers was chosen (Table 1) that should have amplified a region of ≈ 404 bp in susceptible individuals. However, this primer set amplified DNA in the susceptible and resistant individuals producing singlet, doublet or triplet band patterns; the multiple bands may have been due to the lack of C/G at the 3' end. The singlet band was ≈ 392 bp, the doublet was comprised of a 392 bp and a 345 bp fragment, and the triplet was comprised of a 392 bp, 345 bp, and a 330 bp fragment (Fig. 2).

Twenty-four of the resistant genotypes of the test progeny had a doublet while only five had a triplet band. Twenty-four susceptible genotypes were characterized by a single band and seven plants had a doublet band similar to the resistant plants. The sequences of all bands were highly homologous to the OPO-16C sequence (Fig. 1).

The sequence from the upper resistant marker band (U), which was present in all progeny, had 98.7% homology to the susceptible allele marker (OPO-16C) (Fig. 1). A 2 bp change in the sequence and one unmatched T were observed as the only

Fig. 3. A resistant (R) and susceptible (S) genotype as screened with the RAPD primers OPC-8, OPO-8, OPO-16C, and the SCAR-R1A marker. Arrows indicate the polymorphic markers identified previously as being linked to the *Rpfl* gene. OPO-16C marker is linked in repulsion phase to the *Rpfl* gene. The molecular weight (in bp) is indicated by M. A linkage map is included to indicate the already published RAPD markers (Haymes et al., 1997) and the SCAR-R1 markers distance from the gene in cM.

differences. The middle fragment (M), present in all resistant progeny and in only a few susceptible progeny, had 95% homology when compared to the OPO-16C sequence (Fig. 1). This fragment contained eight unmatched bases of which seven were part of a deletion region and the extra T (position 212). Besides the eight unmatched bp, a total of 7-bp changes between the OPO-16C and middle fragment sequences were observed (Fig. 1). In a comparison of the upper resistant (U) sequence to the middle resistant (M) sequence, the previously observed 7-bp deletion region (position 285-291) and a 5-bp change were observed. The lowest of the three bands (L), present in only some of the resistant progeny and none of the susceptible, contained a 35 bp deletion, plus the same extra T (position 212) (Fig. 1).

CONSTRUCTION OF SCAR PRIMERS TO THE RESISTANT ALLELE OF *Rpfl*. The 7 bp deletion region in the middle band (M) was used for the creation of specific primers linked to the resistant allele. Two SCAR-R1 primer sets were designed to amplify DNA from the resistant plants only. For the SCAR-R1A forward primer, we used the original forward SCAR-S primer. The reverse primer exploited sequence differences at the 3' end related to the 7 bp deletion region (Table 1). For SCAR-R1A, the expected band size was 285 bp (Table 1 and Fig. 2).

The SCAR-R1B forward primer was based on the deletion region and a 2 bp change in the nucleotide sequence at the 3' end of the middle fragment (Table 1). The reverse primer for this SCAR started 24 bp upstream from the original RAPD primer and the expected length of the amplified region was 133 bp (Fig. 2). These two SCAR-R1 primer sets were tested on the F₁ mapping population of 60 plants and each amplified the expected fragment in resistant plants only, except in the two apparently recombinant plants. The SCAR markers were mapped to the same location as the RAPD OPO-16A/B/C markers at 3 cM from the *Rpfl* gene (Fig. 3).

STRAWBERRY GENOTYPES ASSESSED WITH MOLECULAR MARKERS. Genotypes possessing and lacking *Rpfl*, based on screening tests (Tables 2 and 3), were examined with the following molecular markers: RAPD OPO-8A, RAPD OPC-8D, SCAR-R1A, and SCAR-R1B. The RAPD OPO-8A marker correctly assessed 29 of the 34 resistant genotypes as well as all of the susceptible (*Rpfl*) individuals (Tables 2 and 3). The divergent resistant genotypes were 'Perle de Prague' and four CPRO selections: 88218, 88275, 88310, and 89027.

The SCAR-R1A marker correctly identified 23 of the 34 *Rpfl* red stele genotypes (Table 2). Eight of the 11 divergent *Rpfl*

genotypes are interrelated and their loss of linkage can be explained by a single crossover event during meiosis of Md683. In the resulting cultivar, Stelemaster, *Rpfl* is no longer linked to the SCAR but to the alternate allele OPO-16C. Consequently, the SCAR-R1A marker is also absent in descendants of 'Stelemaster', 'Delite', 'Scott', 'Sunrise', 'Tribute', CPRO 88218, CPRO 88310, and CPRO 88312 while the OPO-16C marker is present in these genotypes. The three other SCAR-R1A divergent genotypes were 'Benton', CPRO 77191, and 'Perle de Prague'. SCAR-R1B produced results identical to those of SCAR-R1A (data not presented). In a screen of 53 *rpfl* susceptible genotypes, 'Cambridge Vigour' was the only genotype possessing the SCAR-R1A marker (Table 3).

RAPD OPC-8D, the farthest marker from *Rpfl*, had a total of 16 nonconforming genotypes of the 87 tested. Eleven *Rpfl* genotypes did not carry the marker ('Annapolis', 'Arking', 'Delite', 'Linn', 'Tribute', 'Perle de Prague', 'Yalova-4', 'Yalova-15', CPRO 88239, CPRO 88246, and CPRO 88275) (Table 2). A single crossover event in one of the parental genotypes ('Cengelköy') or an earlier ancestor could account for the loss of the marker in the latter five. The absence of the marker in 'Arking' may be explained by its absence in the resistant parent 'Delite'. Out of the 53 susceptible genotypes, five contained the OPC-8D marker, which is explained by crossover events (Table 3).

ADDITIONAL GENOTYPES. Another six genotypes, whose resistance was unknown, were tested for the presence or absence of *Rpfl* markers (Table 4). Based upon the molecular data, five of these genotypes were classified as susceptible while the sixth, 'Olympus', could not be determined since it is a recombinant for OPO-8A and SCAR-R1A. If the crossover occurred between *Rpfl* and SCAR-R1A, then 'Olympus' should possess the gene; however, if the crossover occurred between *Rpfl* and OPO-8A then most likely the gene was lost.

An additional 19 cultivars and 21 CPRO selections, whose parents lacked *Rpfl* as well as the markers, were assessed with OPO-8A and SCAR-R1A (Table 5). As expected, all lacked the markers.

Discussion

Genetic markers represent a useful tool for plant breeding since the presence of genes can be detected at an early stage of plant development without waiting for the phenotypic expression of the gene in the plants. To overcome the disadvantages associ-

Table 4. Reactions of six European and North American strawberry selections with undetermined *Phytophthora fragariae* susceptibility (susc) that were tested with RAPD and SCAR markers linked to the *Rpfl* gene. Genotype *Rpfl* resistance (resist) is predicted based upon the molecular data and upon the ancestry of the selections. Male parents are in bold.

Genotype	Country of origin ²	Parental plants	OPO-16C (susc) ^y	OPC-8D (resist) ^y	OPO-8A (resist)	SCAR-R1A	Predicted <i>Rpfl</i> (resist) ^x
Ettersburg 121	USA	<i>F. chiloensis</i> (Cape Mendocino) x <i>F. vesca</i> cv. Alpine	—	—	—	—	—
Mimek	DK	Unknown x Unknown	—	—	—	—	—
Olympus	USA	Molalla x Columbia	+	+	+	—	?
Puget Beauty	USA	ORUS 1765 (Dorsett x OSC 346 (Narcissa x OSC 59 (Ettersburg 121 x Improved Oregon) x Sparkle	+	—	—	—	—
Totem	CAN	Puget Beauty x Northwest	+	—	—	—	—
Tyee	CAN	Totem x Olympus	+	—	—	—	—

²All plant material was obtained from the USDA National Germplasm Repository, Corvallis, Ore., DK = Germany and CAN = Canada.

ated with RAPD markers, such as irreproducibility among laboratories, RAPD markers have been converted into highly specific SCAR markers (Paran and Micheltore, 1993). Such SCAR markers have proven useful for fingerprinting, marker-assisted selection, and high-resolution mapping (Kaplan et al., 1996; Paran and Micheltore, 1993; Xu et al., 1995).

In this study, a SCAR marker was constructed for the strawberry gene *Rpfl*, which represents one of the major sources of genetic resistance to *P. fragariae*. Initially a SCAR-S primer set was constructed based upon a RAPD marker (OPO-16C₍₄₃₈₎) linked to the susceptibility allele. Previously, we reported that the OPO-08 markers have a relatively high molecular weight and are difficult to score due to a bright monomorphic band of 1700 bp (Haymes et al., 1997). The OPO-16C marker was chosen over

OPO-16A₍₅₁₀₎ and OPO-16B₍₄₅₀₎ due to the intensity of the band compared with the other two markers (Haymes et al., 1997). The SCAR primers amplified DNA of different molecular weights in susceptible as well as resistant plants. The bands in the resistant plants allowed creation of two SCAR markers specific for the resistant *Rpfl* allele (SCAR-R1A and SCAR-R1B), which cosegregated completely with the OPO-16C₍₄₃₈₎ in a test cross. This was expected based upon the sequence similarity of the resistant bands to the susceptible bands.

MAINTENANCE OF THE MARKERS IN STRAWBERRY SELECTIONS. The linkages of the RAPD and SCAR markers to the *Rpfl* gene were maintained in most of the genotypes examined. These genotypes originated from breeding programs in The Netherlands, United States, Canada, Scotland, and Turkey. The linkages

Table 5. Nineteen *Rpfl* susceptible cultivars and 21 CPRO *Rpfl* susceptible selections in which both parents have been tested with the nearest flanking markers (OPO-8A and SCAR-R1A) to the *Rpfl* gene and shown to be absent for them. Male parents are in bold.

Genotype	Country of origin ^c	Parental plants
Blomidon	CAN	K72-4 [(Micmac x (Guardian x Tioga)] x Holiday
Bounty ^y	CAN	Jerseybelle x Senga Sengana
Elsanta ^y	NL	Gorella x Holiday
Fairland ^y	USA	Aberdeen x Fairfax
Glooscap ^y	CAN	Micmac x Bounty
Governor Simcoe ^y	CAN	Holiday x Guardian
Induka ^y	NL	Puget Beauty x Senga Sengana
Korona ^y	NL	Tamella x Induka
Polka ^y	NL	Induka x Sivetta
Primella ^y	NL	Gorella x Macherauch's Frühernte
Raritan ^y	USA	Redglow x Jerseybelle
Sivetta ^y	NL	Redgauntlet x Gorella
Sparkle ^y	USA	Fairfax x Aberdeen
Tago ^y	NL	Gorella x Talisman
Tamella ^y	NL	Talisman x Gorella
Temple ^y	USA	Aberdeen x Fairfax
Tenira	NL	Redgauntlet x Gorella
Tioga	USA	Lassen x Cal 42.8-16
Valeta ^y	NL	Sivetta x Holiday
CPRO 87011	NL	Elsanta x Cambridge Favourite x Induka
CPRO 87041 ^y	NL	Redchief x Sivetta x Korona
CPRO 88030 ^y	NL	(Sivetta x Holiday) x Korona x Lambada
CPRO 90074 ^y	NL	(Induka x Sivetta) x Earliglow x (Sivetta x Holiday) x Korona
CPRO 91008 ^y	NL	Elsanta x MDUS 3184
CPRO 91012 ^y	NL	Elsanta x Chandler
CPRO 91020 ^y	NL	Elsanta x Chandler
CPRO 91023 ^y	NL	Elsanta x Selva
CPRO 91033 ^y	NL	Korona x MDUS 3184
CPRO 91046 ^y	NL	Korona x Chandler
CPRO 91058 ^y	NL	Korona x Selva
CPRO 91066 ^y	NL	Korona x Selva
CPRO 91067 ^y	NL	Korona x Selva
CPRO 91070 ^y	NL	(Bogota x Sivetta) x Elsanta x MDUS 3184
CPRO 91088	NL	(Bogota x Sivetta) x Elsanta x Chandler
CPRO 91100 ^y	NL	(Bogota x Sivetta) x Elsanta x Selva
CPRO 91113 ^y	NL	Holiday x Chandler
CPRO 92033	NL	Sivetta x Holiday
CPRO 92041 ^y	NL	Elsanta x Polka
CPRO 92074	NL	Polka x Chandler
CPRO 92075	NL	Polka x Selva

^aPlant material was obtained from CPRO-DLO strawberry collection and breeding program, The Netherlands for all NL selections and the USA selection of 'Tioga'. The other selections from the USA and Canada (CAN) came from the USDA National Germplasm Repository, Corvallis, Ore.

^yOPO-16C is present in these selections.

Fig. 4. Pedigree of CPRO 90025 genotype. Resistant cultivars for *Rpfl* are in bold text while susceptible are in normal text. Genotypes that gave positive results in the test for the presence of RAPD OPO-8A and SCAR-R1 markers are indicated by a box whereas genotypes that lacked the markers are underlined. No box and no underline indicate that the individual was not tested.

were maintained, irrespective of where these selections were bred. The markers should therefore be applicable for marker-assisted selection in breeding programs, phylogeny studies, and cultivar identification. For example, the data indicate that OPO-8A and the SCAR markers have been maintained in many crosses over succeeding generations. This is illustrated by the pedigree of the *Rpfl* selection CPRO 90025 (Fig. 4) in which these markers were maintained through five generations.

The only cultivar in which the presumed presence of *Rpfl* could not be established by any of the markers is 'Perle de Prague'. The origin for this discrepancy could not be determined since its parentage is unknown. The absence of each of the molecular markers may indicate that it carries another gene that has not yet been determined in the strawberry-*P. fragariae* gene-for-gene model, and which is similar to *Rpfl* in respect to its resistance to the relative races (A1-A4, A6-A10, and NS2-NS4) (Van de Weg, 1997) or the present markers may not yet be close enough to detect recombination events in the resistance gene reported.

MARKER-ASSISTED SELECTION. The RAPD and SCAR markers assessed can be used for marker-assisted selection aimed at the efficient introgression of *Rpfl* and the pyramiding of resistance genes into new cultivars. Red stele resistance tests are expensive, laborious, time consuming, affected by environmental factors, and suffer from incomplete resistance and epistatic effects among resistance genes (Van de Weg, 1997b). Additionally, in Europe, *P. fragariae* is a quarantine pathogen, therefore requiring special laboratory facilities. In contrast, RAPD and SCAR markers are relatively inexpensive, reliable, and are easily and quickly screened for, in that the marker is either present or absent, intermediate scores not being possible. The present markers may therefore encourage breeding for red stele resistance since their use is economically and technically more feasible than screening by plant/pathogen tests for the resistant phenotype.

Resistance is best determined by the use of flanking markers since this minimizes detection of false positives. In this respect,

OPO-8A and the SCAR markers can be used due to their strong linkage to *Rpfl* (Fig 3). In some genotypes, like 'Stelemaster' and its descendants, RAPD OPO-16C is linked to *Rpfl* instead of the SCAR-R1A. OPO-8A and OPO-16C could then be used as the flanking markers. However, this is only applicable if OPO-16C is absent in the other parent.

Dominant markers such as these SCARs are generally not suitable to distinguish heterozygous from homozygous genotypes. However, the present markers do allow such discernment, since *Rpfl* exhibits disomic segregation (Haymes et al., 1997), and since SCAR-R1A and OPO-16C are alternate alleles. In R x R progenies in which both parental genotypes possess SCAR-R1A as well as OPO-16C, progenies lacking OPO-16C should be homozygous for *Rpfl*. This approach has been followed in a population of 24 progeny of Md683 selfed, of which 7 are predicted to be homozygous resistant, 15 to be heterozygous, and two to be homozygous susceptible for *Rpfl* according to the molecular data (data not presented). These numbers fit the expected 1:2:1 segregation ratio at the 95% level ($\chi^2 = 3.9$).

In pyramiding strategies that are considered for development of durable disease resistance, identification of molecular markers for each desired resistance gene is required. Therefore, efforts are underway to map three more *Rpfl* genes. The results should allow application of efficient selection schemes for pyramiding red stele resistance genes in superior cultivars, provided they map to different chromosomal regions.

PHYLOGENY. The occurrence of *Rpfl* in most cultivars of eastern North America and in some cultivars ('Benton', 'Linn', and 'Hood') from the west coast of the United States, Scotland, and The Netherlands, was not surprising since they all derived their resistance from the oldest known source of *Rpfl*, 'Frith', generally via Md683, a second generation descendent of 'Frith' (Reid, 1952; Scott et al., 1984). Interestingly, *Rpfl* is also present in the Oregon cultivar 'Siletz' and the Turkish cultivars 'Yalova-4' and 'Yalova-15', based on the resistance tests and the molecular markers, although to our knowledge 'Frith' is not in their

ancestry. The probability of a similar resistance gene arising independently is highly unlikely, and therefore we believe that this *Rpf* gene came from a much older common ancestral genotype and that the linkage of the markers was conserved through these earlier generations.

In case of two *Rpf* resistant parents, the markers can sometimes clarify the parental origin of the gene. For instance, 'Tribute' and 'Tristar' may have received *Rpf* from two resistant grand parents, 'Surecrop' and 'Sunrise' (Table 2). In 'Surecrop', *Rpf* is linked to the SCAR-R1A while in 'Sunrise' *Rpf* is linked to OPO-16C. Since 'Tribute' shows linkage to OPO-16C and not the SCAR, the *Rpf* allele should have come from 'Sunrise'. In contrast, the SCAR-R1A marker present in 'Tristar' is due to an *Rpf* allele received from 'Surecrop'. The simultaneous presence of OPO-16C indicates that 'Tristar' also carries an *Rpf* allele of 'Sunrise', thus being homozygous for *Rpf*, or that one of its susceptible grandparents carries this marker.

CULTIVAR IDENTIFICATION. These markers have proven useful also for cultivar identification. Previously two 'Aberdeen' accessions were distinguished by Van de Weg et al. (1997a) based on their resistance to *P. fragariae*. The accession that is most likely to be true to type, namely that of the Scottish Crop Research Institute (SCRI), possesses *Rpf2* and *Rpf3*, whereas it lacks *Rpf1* (Van de Weg, 1997). The other accession came from North Carolina State University (NCSCU) and had the same resistance as MD683. Our molecular tests confirmed these findings. The Scottish accession lacked the *Rpf* flanking markers, while the accession from North Carolina had them.

Conclusions

A reliable SCAR marker (SCAR- R1A) that is linked to the *Rpf* resistant allele was developed from the sequence of a RAPD marker (OPO-16C), which originally cosegregated with susceptibility in the testcross population. The molecular markers SCAR-R1A and RAPD OPO-8A can be used in marker-assisted selection for resistance to *Phytophthora fragariae* var. *fragariae* since they are highly conserved to the *Rpf* gene.

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