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A detailed linkage map around an apple scab resistance gene demonstrates that two disease resistance classes both carry the V_f gene

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Abstract A detailed genetic map has been constructed in apple (*Malus* × *domestica* Borkh.) in the region of the V_f gene. This gene confers resistance to the apple scab fungus *Venturia inaequalis* (Cooke) Wint.. Linkage data on four RAPD (random amplified polymorphic DNA) markers and the isoenzyme marker PGM-1, previously reported to be linked to the V_f gene, are integrated using two populations segregating for resistance to apple scab. Two new RAPD markers linked to V_f (identified by bulked segregant analysis) and a third marker previously reported as being present in several cultivars containing V_f are also placed on the map. The map around V_f now contains eight genetic markers spread over approximately 28 cM, with markers on both sides of the resistance gene. The study indicates that RAPD markers in the region of crab apple DNA introgressed with resistance are often transportable between apple clones carrying resistance from the same source. Analysis of co-segregation of the resistance classes 3A (weakly resistant) and 3B (weakly susceptible) with the linked set of genetic markers demonstrates that progeny of both classes carry the resistance gene.

Key words *Malus* × *domestica* · *Venturia inaequalis* · *Malus floribunda* · Genetic map · Disease resistance

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Introduction

Combining disease resistance with the valuable characteristics of a commercially successful cultivar is a prime objective of most plant breeding programmes. In the domesticated apple (*Malus* × *domestica*) there has been a concerted effort to introgress genes that are derived from wild crab apples and that confer resistance to the apple scab fungus *Venturia inaequalis* (Crosby et al. 1992). This was achieved by modified backcrossing programmes which entailed repeated crossing of resistant progeny with commercial cultivars of *M.* × *domestica*. In 1914, Crandall initiated a cross between cv Rome Beauty and the crab apple *Malus floribunda* (Sieb. ex Van Houtte) clone 821 that possesses the disease resistance gene V_f (Crandall 1926). The first commercial cultivar resulting from this cross was 'Prima', a fourth generation descendent of *M. floribunda* 821 (introduced in 1970). This example demonstrates how slow traditional methods for introgressing genes from related species can be in woody plants with long generation times.

The advent of molecular markers has made the prospect of producing genetic maps which can be used to accelerate plant breeding a reality. In apple, a genetic map containing molecular markers has recently been published (Hemmat et al. 1994) and other maps are being constructed (Gardiner et al. 1994; King 1994). The ability to map the position of agronomically important apple genes has several benefits. Selecting for molecular genetic markers linked to an important gene(s) can be used to accelerate traditional methods of breeding, a technique known as marker assisted selection or MAS (Lande and Thompson 1990; Gianfrancheschi et al. 1994). The confidence that progeny identified by marker assisted selection actually contain the gene of interest can be increased by molecular markers on both sides of the gene (Koller et al. 1994). The same molecular markers can also be used to initiate the construction of a fine structure map around the gene, which is a pre-requisite for map-based positional cloning strategies (Tanksley et al. 1995).

The first successful map-based cloning projects involving plant disease resistance genes were published recently (Martin et al. 1993; Mindrinos et al. 1994). Notwithstanding the difficulties associated with somaclonal variation, gene expression, and new problems such as sense suppression (Napoli et al. 1990), it should be possible to transform such cloned genes into commercial cultivars and thereby confer natural resistance on these cultivars while retaining their unique blend of characteristics. This has not been possible using traditional breeding approaches. The importance of being able to clone disease resistance genes for direct transfer into cultivars is underlined by the recent appearance of races of *V. inaequalis* that can overcome V_f resistance (Parisi et al. 1993; Roberts and Crute 1994).

When combined with the need to continually bring out improved cultivars, the traditional route of introgressing resistance factors is too slow relative to the ability of the fungus to overcome resistance based on single genes. Disease management strategies involving strategic planting of unrelated cultivars in orchards that are designed to slow down the spread of resistant races have been proposed (Gessler and Blaise 1994). However, cloning resistance factors offers one of the few practical strategies that can circumvent the almost inevitable breakdown of single gene resistance. In addition it offers the alternative of pyramiding a series of cloned resistance genes into cultivars to reduce the chance of new isolates being able to overcome a single resistance gene in isolation. Molecular markers can also improve the efficiency of incorporating quantitatively inherited (multi-component) disease resistance systems that are often described as being more durable than single resistance genes (Dayton et al. 1983). However, the latter strategy still involves several generations of backcrosses to select the rare recombinants containing the multiple resistance factors and all of the vital fruit and plant characteristics required in a modern cultivar. Cultivars with quantitatively inherited resistance are not favoured in traditional breeding strategies since the effective resistance is difficult to transmit to progeny.

The scab resistance in the crab apple *M. floribunda* 821 is thought to consist of a single major effect gene (V_f) and a number of modifying genes (Dayton et al. 1983). The resistance evident in this crab apple produces a classic pinpoint or hypersensitive reaction in response to inoculation with *V. inaequalis* races unable to overcome the resistance. When the V_f resistance is passed on to progeny, a range of reactions from hypersensitive responses (HR) to weakly susceptible reactions are evident (Hough et al. 1953). Chevalier et al. (1991) carried out detailed scanning electron microscopy and histological studies and defined five classes of reactions. These were: class 1 – a typical HR pin-point reaction, resistant; class 2 – chlorotic lesions with some slight necrosis, resistant; class 3A – necrotic lesions with occasional slight sporulation, weakly resistant; class 3B – clearly sporulating necrotic and chlorotic lesions, weakly susceptible; class 4 – abundant sporulation with no chlorotic or necrotic response, susceptible. Classes 1, 2 and 3A are generally accepted as being resistant. However, there has been a degree of doubt that the weakly susceptible progeny in class 3B actually carry the V_f resistance gene and whether 3B progeny should be classed as susceptible.

In this manuscript we present a detailed linkage map around the V_f resistance gene using RAPD and isoenzyme markers. We integrate the position of several previously published markers which were reported to be linked to V_f (Koller et al. 1994; Manganaris et al. 1994; Tartarini 1994; Yang and Kruger 1994) into a single map around this gene and have identified several additional linked RAPD markers by bulked segregant analysis (Michelmore et al. 1991). By analyzing the segregation of the markers linked to V_f in class-3A and -3B progeny, we have also been able to address the question of whether both of these classes contain the resistance allele of this gene.

Materials and methods

Segregating families

Two sets of progeny segregating for scab resistance were used in this study. One set of 98 progeny were derived from a cross between cv 'Granny Smith' (susceptible) and A679-2 (a V_f heterozygote derived from a cross between cv 'Worcester' and A363-38). This progeny family is referred to as family 1. A set of 160 progeny (referred to as family 2) were chosen from amongst 658 seedlings of a cross between cv 'Royal Gala' (susceptible) and A172-2 (a V_f heterozygote derived from a cross between cv 'James Grieve' and OR33T90). Approximately equal numbers of progeny were selected from each of the three main classes of plant/fungal interactions segregating in this family (3A, 3B and 4) in order to determine if the V_f gene segregates with both classes 3A and 3B. The three groups selected consisted of 49 weakly resistant (class 3A), 55 weakly susceptible (class 3B), and 56 susceptible (class 4) progeny.

DNA isolation

DNA was isolated from young apple leaves less than 1.5 cm long. Leaves were frozen and stored at –70°C before extraction. Two leaves were placed in a plastic bag and 2 ml of DNA extraction buffer (140 mM Sorbitol, 220 mM Tris-HCl pH 7.5, 20 mM EDTA, 0.8 M NaCl, 0.8% w/v Cetyl trimethylammonium bromide, 1% w/v N-Lauroylsarcosine, 1% w/v Polyvinyl-pyrrolidone 4000) was added and the bag was heat-sealed. The leaves were ground to a homogeneous pulp by applying a Teflon roller to the sealed bag placed on a flat surface. A 1.6-ml sample of the pulp was extracted with 400 µl of chloroform:octanol (24:1 v/v) for 30 min at 65°C and centrifuged in a 2-ml Eppendorf tube at 15 000 rpm for 10 min. The aqueous layer was collected and DNA was precipitated by adding 1.0 ml of ice-cold isopropanol. DNA was recovered by centrifugation at 12 000 rpm for 5 min, the pellet washed twice with 70% ethanol, dried under vacuum and re-suspended in sterile distilled water.

PCR amplification conditions and RAPD Primers

PCR amplifications were performed in a 12.5-µl reaction mix containing 10 mM Tris-HCl pH 8.8, 50 mM KCl, 1.5 mM MgCl2, 0.01% gelatin, 1.5% formamide, 0.1 mM dNTP, 0.2 µM of each primer, 1 ng of apple DNA and 1.25 U of *Taq* polymerase (Stratagene, La Jolla, Calif., USA, or Life Technologies, Gaithersburg, Md., USA). The amplification reactions were overlaid with 14 µl of paraffin. DNA was amplified in a Perkin Elmer Cetus DNA Thermal cycler with the following programme: 4 min at 94°C, 40 cycles of 1 min at 94°C, 1 min at 40°C and 2 min at 72°C, a final extension cycle of 10 min

at 72°C. RAPD products were loaded on 1% UltraPure/1% NuSieve GTG agarose gels (Life Technologies and FMC Bioproducts, Rockland, Me., USA) or 0.9% Sepharide TM (Life Technologies) in TAE and separated by electrophoresis at 4 V/cm for approximately 5 h. After staining gels with 0.35 μg/ml of ethidium bromide, RAPD amplification products were visualised under UV light. PCR product sizes were estimated using a 100-bp DNA standard ladder (LifeTechnologies). RAPD primers were obtained from Operon Technologies Inc. (Alameda, Calif., USA). The DNA sequences of the RAPD primers used in this study are: OPA15 5′-TTC CGA ACC C-3′; OPC09 5′-CTC ACC GTC C-3′; OPD20 5′-ACC CGG TCA C-3′; S22 5′-CGT CGT GGA A-3′; OPAM19 5′-CCA GGT CTT C-3′; OPM18 5′-CAC CAT CCG T-3′; OPU01 5′-ACG GAC GTC A-3′; OPH01 5′-GGT CGG AGA A-3′; OPR16 5′-CTC TGC GCG T-3′. RAPD markers are abbreviated by adding subscripts to the primer identification, e.g. $OPA15_{900bp}$ designates the 900-bp fragment amplified by primer OPA15.

Bulked segregant analysis

The density of molecular markers in the V_f region was increased by bulked segregant analysis (BSA) using RAPD molecular markers (Michelmore et al. 1991). Two resistant and two susceptible bulked segregant progeny pools, each containing 12 progeny, were constructed for families 1 and 2. Any primers showing RAPD products present in the resistant bulked segregant pools and absent from the susceptible pools were screened across all of the individual progeny from both of the populations.

Isoenzyme analysis

Progeny from family 1 were analysed and scored for segregation of the isoenzyme marker PGM-1 according to previously published methods (Weeden and Lamb 1987; Manganaris et al. 1994). The V_f-resistant parent A679-2 is heterozygous at this isoenzyme locus.

Scab resistance phenotyping

Family 1 was phenotyped by scoring the severity of natural tree infections on a 5-point scale from 0 (very resistant) to 4 (very susceptible) in the orchard. The scale was defined as follows: 0 – no infected leaves; 1 – very few infected leaves; 2 – a few infected leaves on several shoots; 3 – several infected leaves on most shoots; 4 – many infected leaves on all shoots. The problem of inoculation escape was overcome by using phenotype data from one year (1993) when scab infection was particularly serious. These data were backed up by 2 additional years of field screening. For the purpose of BSA, only trees which scored as 0 or 1 in all 3 years were considered as field resistant. Family 2 was phenotyped by artificial inoculation in the glasshouse. Ten monoconidial isolates of *V. inaequalis* collected from scab lesions from apple orchards around New Zealand were grown on 4% potato dextrose agar (BBL, Becton Dickinson Microbiology Systems, Cockeysville, USA). A conidial suspension was prepared by growing a mixture of the ten isolates on susceptible seedlings in the glasshouse, rinsing the infected leaves in water and adjusting the conidial concentration to 4×10^5 /ml. Conidial inoculum was applied to seedlings at the 3–4 leaf stage with an electrical air brush (Wagner Pro-Painter, model 320 W). Subsequent to inoculation, seedlings were incubated for 2 weeks at 20°C±2°C (day temperature) and 16°C±2°C (night temperature) at a relative humidity (RH) of about 95% (RH controlled by a Microcool/Environmist fog system, Environmental Engineering Concepts Inc., Palm Springs, USA). Disease severity was assessed and progeny were assigned to the resistance classes described by Chevalier et al. (1991). Examples of the three main resistance classes segregating in family 2 are presented in Fig. 1.

Map construction

Genetic map distances and gene orders were computed with the program JoinMap Version 1.4 (Stam 1993) using the Kosambi mapping function. The Kosambi mapping function assumes a degree of interference between cross-overs in meiosis. The critical LOD score for linkage groups was set at 3.0 and the critical LOD score for mapping was set at 0.05. The two maps around the V_f gene produced from the segregation data of families 1 and 2 were merged using the map joining function.

Results

Disease resistance screen and isoenzyme analysis

Progeny from family 1 and family 2 were scored for scab resistance as described in Materials and methods. In family 1 approximately 97% of the trees scored either as 0 (49 progeny) or 4 (46 progeny) in the field screen; the remaining progeny scored as 1, 2 or 3 (one progeny in each). In family 2 the segregation numbers were as follows: class 1 – no progeny; class 2 – 1 progeny; class 3A – 53 progeny; class 3B – 232; class 4 – 372 progeny. This segregation pattern does not fit the simple 1:1 ratio expected of a single gene. If classes 2, 3A and 3B are all added together and considered resistant, the chi-square value is 11.24 for a 1:1 ratio (indicating a probability of less than 0.001 of occurring by chance). A likely explanation for this deviation from 1:1 will be provided in the Discussion. Isoenzyme analysis was carried out for the PGM-1 marker using progeny from family 1 only; this marker was not heterozygous in the other family. The *c* and *d* alleles of PGM-1 in family 1 segregated in a 1:1 manner (χ^2 =1.39).

Molecular marker analysis

DNA from progeny of families 1 and 2 were analyzed for the segregation of RAPD markers detected with the primers S22 (Hemmat et al. 1994), OPD20 (Yang and Kruger 1994), OPA15 (Durham and Korban 1994), OPU01 (Koller et al. 1994), OPAM19, OPC09 (Tartarini 1994) and with RAPD primers we have identified by bulked segregant analysis (see below). The RAPD patterns with DNA from the four parents of the two segregating families and the crab apple *M. floribunda* clone 821 are displayed in Fig. 2. Bulked segregant progeny were constructed based on the resistance phenotype of progeny. After linkage data for the RAPD markers $OPA15_{900bp}$ and $OPD20_{500bp}$ were available the bulked segregant pools were re-constructed to ensure that all progeny in the resistant pools also possessed these markers and that the progeny in the susceptible pools were negative for these markers. A total of 350 RAPD 10-bp primers (Operon Technologies Inc.) were screened across both sets of progeny bulked segregant pools. Three further putatively linked RAPD markers ($OPM18_{900bp}$, $OPH01_{1100bp}$ and $OPR16_{400bp}$) were identified by BSA, and both progeny families were analysed with these new markers to confirm linkage.

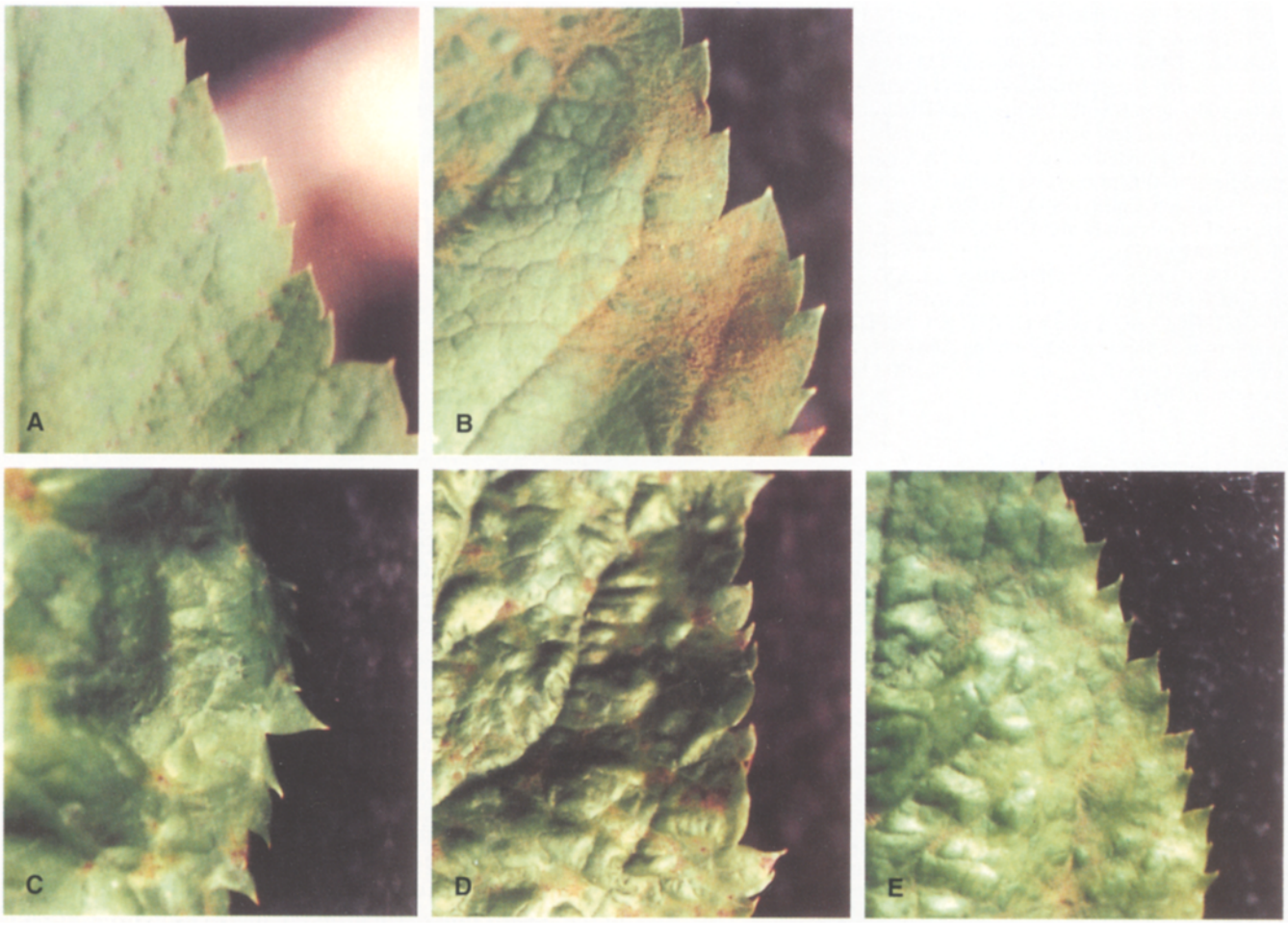

Fig. 1A–E Resistance classes in glasshouse screening. Resistance phenotype of apple leaves, inoculated as described in the Materials and methods scab resistance phenotyping section, demonstrating resistance (**A**), susceptibility (**B**) and intermediate classes of resistance (**C–E**). **A** Class-1 resistance reaction. **B** Class-4 reaction (fully susceptible). **C** Class-2 resistance reaction. **D** Class-3A resistance reaction. **E** Class-3B resistance reaction. All except one of the progeny in family 2 were class 3A, class 3B or class 4

The evidence for linkage (pairwise recombination data) between V_f, RAPD markers, and isoenzyme marker PGM-1 is presented in Table 1, together with a summary of previous information reported for five of these markers. The pairwise recombination data for the three new markers are also presented in Table 1. Marker $OPM18_{900bp}$ was also detected by Koller et al. (1994). Chi-square values indicated that, except for the segregation of $OPC09_{900bp}$ and $OPH01_{1100bp}$ in family 1 which segregate 3:1 and the segregation of scab resistance in family 2 (see below), the segregation of all other markers fit a 1:1 ratio (all χ^2 values ≤ 2.61). Chi-square values for the RAPD markers in family 2 were calculated by taking into account the fact that three groups of progeny were pre-selected for analysis based on their resistance phenotype. If a RAPD marker is linked to the resistance phenotype, this pre-selection would be expected to skew the resulting proportion of progeny from which the RAPD markers can be amplified. If these markers are segregating 1:1 and are linked to resistance, the expected number of progeny from which the RAPD marker can be amplified would be equivalent to the number of resistant progeny (104). The expected number not able to amplify the marker would be equivalent to the number of susceptible progeny (56). Genetic linkage data were processed by the program JoinMap (Stam 1993). The genetic maps in the V_f region for family 1 (A679-2 map), family 2 (A172-2 map), and an integrated map produced by merging the two maps using JoinMap, are presented in Fig. 3. There is a great deal of consistency between the maps produced from the different populations (Fig. 3A and B) and their similarity to the V_f joinmap (Fig. 3C). The only change in the order of the markers is for $OPU01_{400bp}$ which is present on a different side of V_f in the two populations. If the position of PGM1 is set at 0 cM, the relative positions of the markers on the V_f joinmap are: PGM1 – 0 cM; $OPAH01_{1100bp}$ – 6.6 cM; V_f – 11.3 cM; $OPU01_{400bp}$ – 13.6 cM; $OPM18_{900bp}$ – 14.7 cM; $OPA15_{900bp}$ – 20.9 cM; $OPD20_{500bp}$ – 23.0 cM; $OPR16_{400bp}$ – 25.1 cM; $OPC09_{900bp}$ – 28.1 cM.

The data presented in Table 1 demonstrate for the first time that the two new RAPD markers we found by BSA, $OPAH01_{1100bp}$ and $OPR16_{400bp}$, are linked to V_f. In addi-

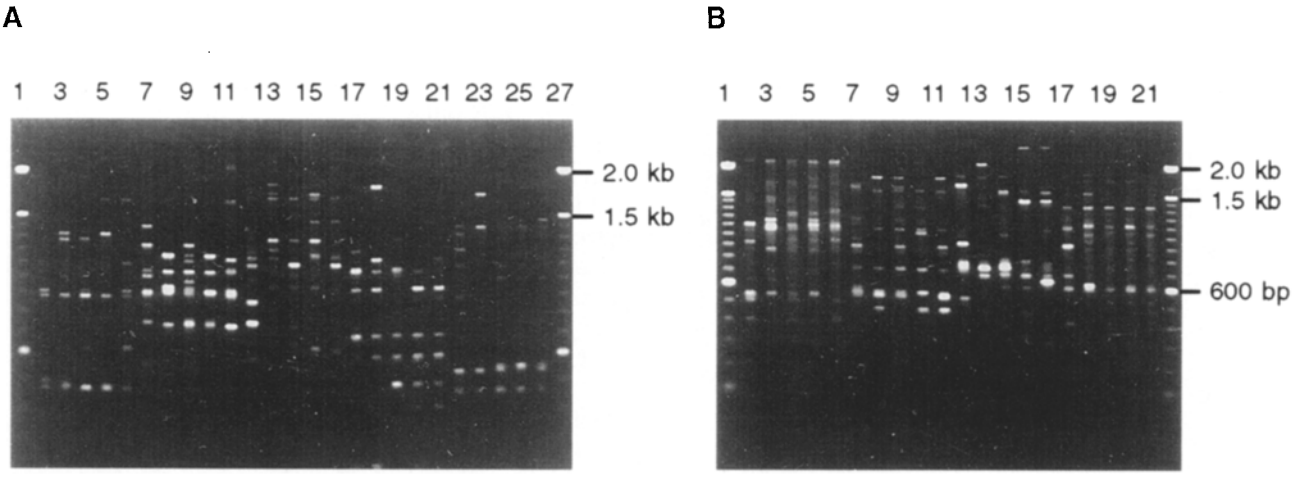

Fig. 2A, B RAPD profiles of parent apple clones. RAPD product profiles of the original *M. floribunda* clone carrying the V_f gene (clone 821) and the four parents used in mapping families 1 and 2. PCR reactions were carried out as described in the Materials and methods section, and RAPD products were separated on 0.9% Sepharide TM (LifeTechnologies). **A** RAPD profiles with the 10-bp primers OPA15 (*lanes 2–6*), OPC09 (*lanes 7–11*), OPH01 (*lanes 12–16*), S22 (*lanes 17–21*) and OPAM19 (*lanes 22–26*). A 100-bp DNA ladder (-Life Technologies) was loaded in *lanes 1 and 27*. Template DNA used in the PCR amplifications was as follows: *M. floribunda* clone 821 in *lanes 2, 7, 12, 17 and 22*; cv 'Granny Smith' in *lanes 3, 8, 13, 18 and 23*; clone A679-2 in *lanes 4, 9, 14, 19 and 24*; cv 'Royal Gala' in *lanes 5, 10, 15, 20 and 25*; clone A172-2 in lanes *6, 11, 16, 21 and 26*. **B** RAPD profiles with the 10-bp primers OPD20 (*lanes 2–6*), OPM18 (*lanes 7–11*), OPR16 (*lanes 12–16*) and OPU01 (*lanes 17–21*). DNA ladder (100 bp) was loaded in *lanes 1* and *22*. Template DNA used in the PCR amplifications was as follows: *M. floribunda* clone 821 in *lanes 2, 7, 12 and 17*; cv 'Granny Smith' in *lanes 3, 8, 13 and 18*; clone A679-2 in *lanes 4, 9, 14 and 19*; cv 'Royal Gala' in *lanes 5, 10, 15 and 20*; clone A172-2 in *lanes 6, 11, 16 and 21*

Table 1 Recombination distances (cM) between V_f and linked markers – a comparison with previously published data

Marker		Distance to V_f [a]	Population details	No. of progeny	Disease screen	Reference
PGM-1		8 (3)	Pool of four populations[b]	197[b]	Field or greenhouse screened[c]	Manganaris et al. 1994
		13 (4)	'Granny Smith' × A697-2^R	98	Field screen	This publication
OPD20	500 bp[d]	25 (8)	81/19-35^R × 'Margol'	28	Not provided	Yang and Kruger 1994
		20 (3)	81/19-35^R × 87/7-10	158	Not provided[e]	Yang and Kruger 1994
		12 (3)	'Granny Smith' × A679-2^R	98	Field screen	This publication
		11 (2)	'Royal Gala' × A172-2^R	160	Greenhouse screen	This publication
OPC09	900 bp	12 (4)	'Prima'^{R} × 'Summered'	[f]	[f]	Tartarini 1994
		14 (3)	'Royal Gala' × A172-2^R	160	Greenhouse screen	This publication
OPA15	900 bp	19 (4)	'Granny Smith' × A679-2^R	98	Field screen	This publication
		11 (2)	'Royal Gala' × A172-2^R	160	Greenhouse screen	This publication
		Not defined	Not a segregating populations	–	–	Durham and Korban 1994
OPU01	400 bp	19.7[g]	'Idared' × *M. floribunda* 821^R	59	Greenhouse screen	Koller et al. 1994
		2 (1)	'Granny Smith' × A679-2^R	98	Field screen	This publication
		5 (2)	'Royal Gala' × A172-2^R	160	Greenhouse screen	This publication
OPM18	900 bp	2 (1)	'Granny Smith' × A679-2^R	98	Field screen	This publication
		6 (2)	'Royal Gala' × A172-2^R	160	Greenhouse screen	This publication
		10.6[g]	'Idared' × *M. floribunda* 821^R	59	Greenhouse screen	Koller et al. 1994
OPH01	1100 bp	10 (2)	'Royal Gala' × A172-2^R	160	Greenhouse screen	This publication
OPR16	400 bp	14 (3)	'Granny Smith' × A679-2^R	98	Field screen	This publication
		13 (3)	'Royal Gala' × A172-2^R	160	Greenhouse screen	This publication

[a] Standard error given in brackets, recombination percentage and standard errors have been rounded to the nearest significant decimal point
[b] Results from four segregating populations were pooled including 'Jonathan' × A849/7^R(37 progeny), 'Idared' × A679/12^R (58 progeny) 'Prima'^{R} × Spartan' (63 progeny) and 'Liberty'^{R} × Royal Gala' (39 progeny). Intermediate resistance progeny were excluded from the linkage estimation
[c] Field resistance phenotype screens carried out on the 'Jonathan' × A849/7^R population and the 'Idared' × A679/12^R population were not performed in an ideal environment
[d] Our estimate of the size of the OPD20 V_f-linked amplification product is 500 bp as opposed to the 600 bp product quoted by Yang and Kruger (1994)
[e] Linkage analysis includes some plants with the resistance phenotype undefined, which could change the distance between the resistance gene and the marker
[f] Details regarding the screening technique and number of progeny in the family are not published
[g] No standard error provided
[R] Denotes the resistant parent

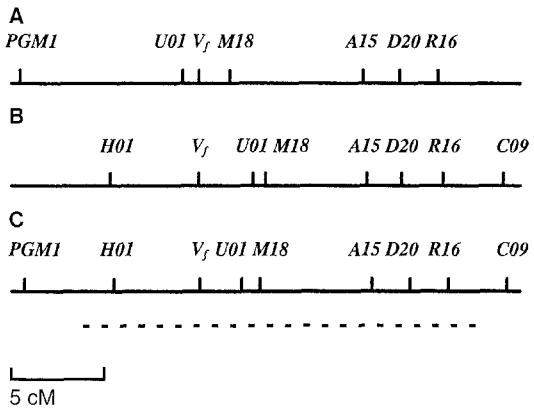

Fig. 3A–C Genetic maps of the region around the V_f resistance gene. **A** Genetic map of the V_f region in family 1 (clone A679-2). **B** Genetic map of the V_f region in family 2 (clone A172-2). **C** Combined genetic map (joinmap) of the V_f region formed by merging the maps for families 1 and 2. The *dotted line* below the map indicates the molecular markers which are present in the crab apple *M. floribunda* clone 821. The molecular markers missing from the map of family 1 (**A**) do not necessarily indicate that they are not present in clone A679-2 but rather that we have not been able to map these markers in this family

tion they show that the $OPA15_{900bp}$ marker is also linked to V_f. Durham and Korban (1994) found that this marker is amplified with *M. floribunda* 821 DNA, and DNA from only some of the scab-resistant Co-op selections derived from this clone. They proposed that it was likely that in these Co-op selections the region carrying this marker was introgressed from *M. floribunda* but that it is not closely linked to V_f. Our results clearly demonstrate that $OPA15_{900bp}$ is linked to V_f at a recombination frequency of approximately 10%. The size estimation of the OPD20 RAPD marker that segregates with the resistance gene in our populations is 500 bp as opposed to the 600 bp quoted by Yang and Kruger (1994). We have assumed that $OPD20_{500bp}$ and $OPD20_{600bp}$ are the same marker and refer only to the former.

There is a high density of markers in the 900-bp region with primer OPC09 in family 1, and a 3:1 segregation ratio of the 1100-bp product with primer OPH01 in this family suggests that there are two products of the same size which are both derived from clone A679-2. Therefore, it was not possible to score definitively the segregation of the $OPC09_{900bp}$ and $OPH01_{1100bp}$ RAPD markers in this family and these results are not presented in Table 1. We did not find any segregating RAPD PCR products that are linked to V_f in the two populations with primer S22 and therefore we could not place this marker on our V_f map. The data of Hemmat et al. (1994) indicate that the locus defined by this marker should be linked to V_f since it is closely linked to the isoenzyme PGM-1; however, the S22 RAPD marker has not yet been shown to be *cis*-linked to the V_f gene. Tartarini (1994) found a 2200-bp marker with the primer OPAM19 which appeared to be linked to V_f, but we did not find a similar marker that segregated with V_f resistance in either family 1 or family 2 and, therefore, could not place the $OPAM19_{2200bp}$ marker on our V_f map.

When the linkage data from the two different phenotype screens (field resistance screen on family 1 and glasshouse screen on family 2) were compared no significant differences in map distances between the V_f resistance gene and RAPD markers common to both populations could be detected. In the glasshouse screen the obvious plant reactions in the resistance classes mean that there was little possibility of progeny being incorrectly scored as resistant due to inoculation escape.

Class-3A versus class-3B resistance

Progeny showing class-3A interactions with *V. inaequalis* are generally considered to be resistant. There is uncertainty whether progeny showing class-3B interactions carry the V_f resistance gene or if they carry other factors that confer a partial resistance. In order to determine if both classes 3A and 3B behaved as being closely linked to the markers linked to V_f in our study, the following analysis was performed separately on two sets of progeny from family 2. Linkage analysis was performed on one set of progeny where all of the class-3B progeny were excluded and a second set of progeny where all of the 3A progeny were excluded (Table 2). This analysis did not detect any statistically significant difference between the linkage of the two classes and the molecular markers linked to the resistance gene.

Discussion

Linkage data

We have integrated mapping data on linkage to the V_f gene from four different sources (Koller et al. 1994; Manganaris et al. 1994; Tartarini 1994; Yang and Kruger 1994) using data from two progeny families. In addition we present the first evidence of linkage for three new markers ($OPA15_{900bp}$, $OPH01_{1100bp}$ and $OPR16_{400bp}$). The data presented provide the first detailed genetic map around the V_f gene with markers on both sides of the gene.

It is noteworthy that several of the RAPD markers which are linked to the resistance gene appear to be amplifiable in more than one cultivar derived from a common resistant ancestor. Presumably, a cross-over event has not yet occurred between these RAPD markers and the introgressed resistance gene in these cultivars. This suggests that RAPD markers in the introgressed chromosomal region of resistant cultivars may often be transportable. For example, the RAPD marker $OPA15_{900bp}$ can still be amplified in a number of cultivars including the four Co-op cultivars reported by Durham and Korban (1994) and both of the resistant cultivars used in this analysis (A679-2 and A172-2). The data suggest that quite sizable sections of introgressed *M. floribunda* DNA are still present in these cul-

Table 2 Comparison of recombination data between V_f and RAPD markers in 3A and 3B progeny

Progeny set[a]	RAPD marker recombination				
	$OPC09_{900bp}$[b]	$OPD20_{500bp}$	$OPA15_{900bp}$	$OPU01_{400bp}$	$OPH01_{1100bp}$
All progeny, classes 3A, 3B, 4	14.4 (2.8)[c]	10.6 (2.4)	11.3 (2.5)	5.0 (1.7)	10.1 (2.4)
Subset A, classes 3A, 4	16.2 (3.6)	10.5 (3.0)	11.4 (3.1)	7.6 (2.6)	11.5 (3.1)
Subset B, classes 3B, 4	15.3 (3.4)	12.6 (3.2)	13.5 (3.2)	5.4 (2.7)	9.0 (2.7)

[a] The data presented in this table are derived from pairwise recombination estimates. The three progeny data sets are not independent since all contain data from class-4 progeny. To compare the difference between 3A and 3B progeny consider that the estimates for 3A and 3B are averages of the form 1/2*(X_3A+X_4), 1/2*(X_3B+X_4), where X_3A, X_3B and X_4 are recombination percentages between the marker and the resistance gene locus. The differences observed are the difference of two expressions such that 1/2*(X_3A+X_4)–1/2*(X_3B+X_4)=1/2*(X_3A–X_3B). The calculated difference is therefore half of the actual difference between 3A and 3B. Doubling the difference between the calculated recombination frequencies gives values of 5.0 ($OPH01_{1100bp}$), 4.4 ($OPU01_{400bp}$), 4.2 ($OPD20_{500bp}$ $OPA15_{900bp}$) and 1.8 ($OPC09_{900bp}$), which are not significant at $P<0.05$

[b] The data sets for the different markers are also not entirely independent since they are genetically closely linked to each other

[c] Recombination percentage with standard error in brackets

tivars and that there is sufficient homology in the introgressed region for meiotic crossing-over to occur. All of the markers in an 18.5-cM region from $OPH01_{1100bp}$ to $OPR16_{400bp}$ appear to be derived from *M. floribunda* clone 821. The $OPC09_{900bp}$ marker was probably derived from cv 'Rome Beauty' and became *cis*-linked to the V_f gene after a meiotic cross-over event between the two loci. Clone A679-2 has retained at least 14 cM of *M. floribunda*-introgressed DNA whereas clone A172-2 has retained at least 18 cM.

It should be practicable to use the RAPD markers most closely flanking the resistance gene in MAS. The presence of markers on both sides of the V_f gene should efficiently tag resistance in crosses using cultivars carrying the identical resistance allele. Genetic markers which are outside the introgressed region would be valuable for assessing if the introgressed region causes any recombination suppression. Such markers could be used to compare the genetic distance between the outside markers in crosses with, or without, the introgressed DNA.

The RAPD marker $OPA15_{900bp}$ co-segregates with resistance, as well as all of the other markers linked to resistance, and therefore clearly behaves as being linked to V_f. Durham and Korban (1994) had previously found this marker to be present in *M. floribunda* 821 and only 4 out of 15 scab-resistant selections derived from this crab apple (with two of the selections containing $OPA15_{900bp}$ being closely related). They proposed that this probably indicates that the marker is not closely linked to V_f. They cautioned that segregation data would be needed for a proper assessment of linkage. Our results using suitable families demonstrate that $OPA15_{900bp}$ maps approximately 10 cM from the resistance gene. That the marker was only present in 4 out of 15 scab-resistant selections can be explained by the fact that all of the Co-op selections are the result of at least four or five meiotic cycles during their respective modified backcross programmes. There has therefore been ample opportunity for cross-overs to occur between the V_f gene and $OPA15_{900bp}$. In theory, 33–60% of resistant selections which have gone through five meiotic events would be expected to have retained a marker which is between 10 cM and 20 cM from the resistance gene. This proportion fits reasonably well with the proportion of resistant selections identified in the analysis of Durham and Korban (1994) and the present analysis (the $OPA15_{900bp}$ marker can be amplified in 6/17 cultivars). Only two of the markers previously shown to be linked to V_f by other groups, S22 and $OPAM19_{2000bp}$, did not appear to segregate in either of our families and could not be placed on the map.

Genetic map distances were computed using the Kosambi mapping function which assumes interference between cross-overs. However, further data will be needed to determine if the Kosambi or Haldane mapping function (which assumes no interference) gives the best genetic map approximation in apple. The only difference between the maps constructed using the Haldane function was a slightly greater distance between most of the markers (data not shown).

Class-3B progeny

The phenotype scoring system devised by Chevalier et al. (1991) differentiates between weak resistance (class 3A) and weak susceptibility (class 3B). Even if host resistance reactions are evident, the development of the fungus and the sporulation which clearly occurs in weakly susceptible progeny (class 3B) are close to the fully susceptible symptoms in class-4 progeny. It is therefore important to establish whether or not class-3B progeny actually carry the resistance gene. The fact that there was no significant difference in the co-segregation of the markers linked to V_f with class-3A and class-3B symptoms clearly indicates that class-3B progeny carry the V_f resistance gene. This analysis demonstrates how molecular markers which are linked to disease resistance genes can be used to analyse the nature of the resistance conferred by the resistance gene(s).

Such linked markers and a complete genetic map can then be used to investigate if there are one or more additional genes segregating in the population which can account for the different classes of resistance. The reduction from the class-1 resistance evident in the crab apple *M. floribunda* down to classes 3A and 3B in many of the commercial cultivars derived from this crab apple has been explained in various ways, including the loss of other genes closely linked to V_f (Williams and Kuc 1969). Evidence from the present study indicates that sizable sections of introgressed DNA from *M. floribunda* (as large as 18 cM) are still present in third and fourth generation descendants which display a reduced resistance phenotype (class 3A and 3B). This fact makes the hypothesis that the loss of closely linked genes causes reduced resistance improbable. It is more likely that the reduction in resistance is due to the loss of resistance-modifying genes (unlinked or loosely linked to V_f) by segregation. With the position of V_f defined, this alternative hypothesis can now be addressed.

Resistance phenotyping

Accurate mapping of disease resistance genes relies on generating high quality data on the resistance/susceptible phenotype of progeny. We used glasshouse and field screens and detected no significant differences in map distances between the V_f resistance gene and RAPD markers common to both populations. Perhaps the greatest risk of mis-scoring progeny in this study involved scoring some of the class-3B progeny as class 4 and *vice versa*. Manganaris et al. (1994) concluded that their group of progeny of intermediate resistance phenotype contained both resistant and susceptible progeny. They specifically excluded progeny of intermediate resistance from their linkage analysis when they found that the segregation of the linked isoenzyme *PGM-1* these progeny was quite different from progeny which could be reliably scored as resistant or susceptible.

In a multipoint linkage analysis, such as the one presented above, the genotype of most progeny with an incorrect resistance classification can be predicted. Most misclassified progeny in family 2 would be identical to progeny which have sustained cross-over events between the resistance gene and the most proximal markers on each side of the gene. Assuming that the marker order in Fig. 2 is correct (see discussion below) only 7 out of the 160 progeny fall into this group (five susceptible and two 3A resistant progeny). This demonstrates that the actual number of misclassified progeny in family 2 as a whole must be quite small (of the order of 5% or less). One or two progeny with a recombination event on each side of the V_f gene could be expected in the entire population (658 progeny) on the basis of chance occurrence. Most of the potentially misclassified progeny are in class 4 (susceptible). The degree of mis-scoring in class-4 progeny alone is approximately 9%, if we assume that these double cross-over progeny in class 4 are incorrectly phenotyped for resistance. This mis-scoring provides a likely reason for the deviation from 1:1 segregation for resistant and susceptible progeny in this family. If the number of resistant and susceptible progeny are adjusted by this correction factor and all of the resistance classes added together (new numbers – 319 susceptible: 339 resistant) the χ^2 value (0.61) would indicate that this fits a 1:1 segregation ratio. If that is the case, some of the molecular markers may be even more closely linked than the data suggests.

In the field screen almost all of the progeny were classified as either 0 (fully resistant) or 4 (fully susceptible) and just two of the plants which appeared resistant in the 1993 season (when scab infection was particularly serious) were subsequently scored as susceptible in one of the other years. Only 2 out of the 98 progeny in family 1 appear to have sustained cross-over events on both sides of the resistance gene, the genotype expected if a progeny with no cross-overs in the V_f region has been misclassified for resistance. This indicates that the degree of misclassification using the field screen must be minimal. The phenotype data from some of the previously published analyses using V_f-linked markers are less certain (see Table 1). In one case the analysis included some progeny which had not been phenotyped (Yang and Kruger 1994). Manganaris et al. (1994) commented that the phenotype screening carried out on two of their populations was not in an ideal controlled environment. The position of the markers presented in some of the earlier publications is consequently less certain than suggested by the standard errors. When compared with the linkage data presented in our study the difference in the calculated positions of these markers with respect to the resistance gene were, in most cases, not statistically significant. The data for the markers $OPM18_{900bp}$ and $OPU01_{400bp}$ (Table 1) suggests that these markers are probably closer to V_f than indicated by the data of Koller et al. (1994). Since these authors did not present statistical data the accuracy of the earlier recombination analysis is uncertain. The linkage of the five markers previously shown to be linked to V_f is confirmed by our analyses. Furthermore, the large number of linked markers also re-inforces the linkage data and gives a more reliable assessment of the placement of these markers with respect to the resistance gene.

Future work

The exact marker order presented in Fig. 3 must still be considered provisional. This was demonstrated by the way that the $OPU01_{400bp}$ RAPD marker maps to different sides of the V_f gene in the two families. This was not surprising given the close linkage between the V_f gene, $OPU01_{400bp}$, and a third marker ($OPM18_{900bp}$). The order of the rest of the markers was well conserved between the two progeny families indicating a degree of robustness. There is the potential for some minor re-shuffling of order between the three other closely linked markers, $OPA15_{900bp}$, $OPD20_{500bp}$ and $OPR16_{400bp}$.

To enable the cloning of the V_f resistance gene, a fine structure genetic map and even more accurate placement

of genetic markers on the map will be needed. To this end we are increasing the size of family 2 and will analyse the linkage data for the rest of the progeny in this segregating population as seedlings become available. We are using the genetic markers placed on the map around V_f to limit the search for further linked markers to the region of the map delineated by outside markers such as $OPC09_{900bp}$. In addition, we are developing AFLP technology (Zabeau 1993; Tanksley et al. 1995) in apple to accelerate the rate of isolation of linked markers by BSA and to increase the density of markers around this resistance gene.

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