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ORIGINAL PAPER

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Identification of molecular markers linked to the mildew resistance gene *Pl-d* in apple

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Abstract Powdery mildew poses a serious problem for apple growers, and resistance to the disease is a major objective in breeding programmes for cultivar improvement. As selective pressure allows pathogens to overcome previously reliable resistances, there is a need for the introduction of novel resistance genes into new breeding lines. This investigation is concerned with the identification of the first set of molecular markers linked to the gene for mildew resistance, *Pl-d*, from the accession 'D12'. As no prior information on the map position or markers for *Pl-d* were available, a bulked-segregant approach was used to test 49 microsatellite primers, 176 amplified fragment length polymorphism (AFLP) primers and 80 random amplified polymorphic DNA (RAPD) primers in a progeny segregating for *Pl-d* resistance, 'Fiesta' (susceptible) × A871-14 ('Worcester Pearmain' × 'D12'). The segregations of the markers identified in the resistant and susceptible bulks were scored in the progeny, then the recombination fractions between *Pl-d* and the most tightly linked markers were calculated and a map prepared. Three AFLP, one RAPD and two microsatellite markers were identified. One AFLP was developed into a sequence-characterised amplified region marker, while the microsatellites CH03C02 and CH01D03 were flanking markers, 7 and 11 recombination units, respectively, from *Pl-d*. Two more distant microsatellites on the same linkage group, CH01D09 and CH01G12, confirmed the orientation of the markers on the linkage group. These microsatellites place *Pl-d* on the bottom of linkage group 12 in published apple maps, a region where a number of other disease resistance genes have been identified.

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Introduction

Increasing the level of resistance to powdery mildew (*Podosphaera leucotricha*) is an important objective for apple breeders, particularly in the UK, where growers may apply up to 17 fungicide sprays each year (Butt et al. 1983). Breeders have concentrated on introducing the resistance genes *Pl-1* from *Malus robusta* and *Pl-2* from *M. zumi* (Knight and Alston 1968) into traditional breeding lines, some of which also carry a low level of polygenic resistance to mildew. Other sources of resistance to mildew, *Pl-w* from 'White Angel' (Gallott et al. 1985; Simon and Weeden 1991), *Pl-d* from 'D12' (Visser and Verhaegh 1980) and *Pl-m* from 'Mildew Immune Selection' ('MIS') (Dayton 1977), have also been introduced into breeding lines at East Malling.

The durability of resistance to disease that relies on a single major gene is limited by the probable development of a virulent race of pathogen, and such resistance has been broken down in many crop species (Parlevliet 1993). Races of mildew have been isolated that are able to overcome the resistances from *M. robusta* and *M. zumi* (Krieghoff 1995), and the breakdown of resistance from 'White Angel' and 'MIS' has also been reported (Lespinasse 1989; Korban and Dayton 1983). Breeding strategies have been developed to 'pyramid' these major gene resistances, into a background of polygenic resistance wherever possible, in order to maximise their durability; the chances of a pathogen developing a race capable of breaking down this multiple gene barrier are therefore greatly reduced (Pederson and Leath 1988).

In apple, although it is relatively easy to combine these genes by intercrossing the previously developed breeding lines, selection of individuals that carry more than one major gene proved to be almost impossible until the advent of linked molecular markers. DNA markers have been reported for *Pl-1* (Markussen et al. 1995), *Pl-2* (Seglias and Gessler 1997; Dunemann et al. 1999; Gardiner et al. 1999), *Pl-w* (Evans and James

2003) and *Pl-m* (Gardiner et al. 2002), and these can be used to pre-select individuals from seedling populations that carry multiple resistance genes.

Bulk segregant analysis (BSA) (Michelmore et al. 1991) is a powerful technique which allows markers to be identified rapidly through the simultaneous screening of two bulks of DNA pooled from two sets of seedlings of contrasting phenotype. It is invaluable for identifying markers for genes such as *Pl-d*, for which no markers or mapping data are available. BSA was used with a combination of three marker methods, AFLPs, RAPDs and microsatellites, to produce a preliminary linkage map of the region around *Pl-d*.

Amplified fragment length polymorphism (AFLP) analysis generates large numbers of polymorphic markers suitable for marker saturation. The markers are highly reproducible and, though labour intensive, the technique requires relatively little DNA (Vos et al. 1995). Random amplified polymorphic DNA (RAPD) analysis also offers the potential of generating large numbers of polymorphic bands, though reproducibility is often a problem. Both AFLPs and RAPDs have been used successfully for the identification of molecular markers linked to the mildew resistances *Pl-w* and *Pl-1* in apple (Evans and James 2003; Markussen et al. 1995). Furthermore, RAPDs and AFLP markers can potentially be converted into sequence-characterised amplified region (SCAR) markers which are more robust and breeder friendly. Microsatellite or simple sequence repeat (SSR) markers are co-dominant, highly polymorphic and robust; they are also readily transferable between breeding progenies and genetic maps, which is generally not the case for AFLPs and RAPDs. The availability of a large number of apple microsatellite primers (Liebhard et al. 2002; Gianfranceschi et al. 1998; Guilford et al. 1997; Hokanson et al. 1998) will allow the position of *Pl-d* to be assigned to a linkage group corresponding to published apple maps.

In this paper we report the first DNA markers for *Pl-d* resistance. This gene for resistance is from 'D12', one of the 'D' series of seedlings (Visser and Verhaegh 1976). 'D12' has been shown to provide a higher level of mildew resistance than *Pl-1* or *Pl-2*, particularly in glasshouse tests (Alston 1983).

Materials and methods

Plant material

A progeny of 272 individuals from the cross made in 1995 of 'Fiesta' × A871-14 ('Worcester Pearmain' × 'D12'), heterozygous for *Pl-d*, was raised and screened for resistance to mildew. A subgroup of 86 individuals was analysed for microsatellite, AFLP and RAPD markers. The progenitors of the progeny, 'Fiesta', A871-14, 'Worcester Pearmain' and 'D12' were also tested.

Additional progenies and varieties

Leaf material from a range of individuals known to carry different genes for mildew resistance was obtained from the gene bank at East Malling in order to test the markers in a range of genetic backgrounds. These included 3760 and 3762 (*M. robusta* open-pollinated seedlings), 3752 and 3753 (*M. zumi* open-pollinated seedlings), 'White Angel' and 'MIS' (which are reported to have single major resistance genes) and 'Worcester Pearmain', one of the parents of the progeny, which has polygenic resistance. The mildew susceptible cultivars 'Fiesta', 'Prima', 'Gloster 69' and 'Florina' were included as further controls.

A progeny combining *Pl-d* with the resistance gene *Pl-w* was also tested to establish the suitability of the markers for multiplexing. E711 (A871-14 × E295-4) combines *Pl-d* and *Pl-w*.

Disease assessment and bulked samples

The seedlings were screened for natural infection for two seasons in the glasshouse, followed by four seasons in the field. The symptoms were scored on a 0–5 scale of symptom severity, using the Pl-GH-1 descriptor developed in the European Apple Genome Mapping Project and described in Evans and James (2003).

DNA bulks were made up from ten individuals selected from the extreme ends of the scale, resistant (score 0, no symptoms) and susceptible (score 4 or 5, severe infection). A further 66 individuals (which showed a clear resistant or susceptible phenotype) were screened, with the most tightly linked markers identified by BSA to establish co-segregation with resistance.

DNA extraction and quantification

DNA was extracted from young leaves, following the SDS/potassium acetate method described by Dellaporta et al. (1983) and quantified against lambda DNA standards on 0.8% agarose gels stained with ethidium bromide. The DNA was diluted to 100 ng/μl and used to make the two bulks. Further dilutions of 10 ng/μl were made for marker analysis.

AFLP analysis and cloning of amplified fragments

The DNA bulks were screened with 176 AFLP primer combinations comprising 128 *Eco*RI/*Mse*I, 24 *Pst*I/*Mse*I and 24 *Kpn*I/*Mse*I primers as described in Evans and James (2003). Primer combinations which detected clear differences between the bulks were tested in ten resistant and ten susceptible individuals, and then in 55 individuals from the sub-population of 86 and the controls if the marker appeared to co-segregate with *Pl-d*.

AFLP fragments found to be tightly coupled with *Pl-d* resistance were excised from the dried gel and incubated overnight at 4°C in 50 µl TE (10 mM Tris, 1 mM EDTA, pH 8.0). Aliquots of the template DNA were re-amplified in two rounds of polymerase chain reaction (PCR), the first using the primers and conditions described for pre-amplification (Ellis et al. 1997; Vos et al. 1995) and the second with the appropriate selective primers as described in Evans and James (2003). The products were cloned into the plasmid pGEM-T Easy Vector (Promega) and then re-amplified alongside the original AFLP reactions to ensure that the correct bands had been cloned (Evans and James 2003). DNA sequencing was performed using pUC/M13 primers on an automated sequencer (Applied Biosystems 3100).

SCAR primers were designed from the sequences, using PRIMER 3 (Center for Genome Research, Whitehead Institute, Mass., USA). Initially the primers were tested in the ten resistant and ten susceptible individuals which made up the bulks, using the PCR conditions described in Evans and James (2003), with annealing temperatures ranging from 5°C above to 5°C below the T_m of the primers. The products were separated on a 1.5% agarose gel in 1× TAE buffer at 90 V for 1 h and visualised under UV light, following staining with ethidium bromide.

SCAR primers which amplified a single, strong product (but did not distinguish between resistant and susceptible individuals when analysed on agarose gels) were used to amplify fragments from susceptible individuals, which were then cloned and sequenced as above. The susceptible sequences were aligned with the resistant sequences (generated from the same SCAR primers), using Megalign (DNAstar, Madison, Wis., USA) and the primer pairs redesigned to exploit the differences between resistant and susceptible sequences. The new primers were tested as above in the subset of 20 individuals; those shown to distinguish the ten resistant and ten susceptible individuals were then tested in the remaining members of the sub-population.

Microsatellite analysis

The DNA bulks were screened with all 30 microsatellite primers described in Guilford et al. (1997) and Gianfranceschi et al. (1998) and 14 primers from Liebhard et al. (2002) (CH01D08, CH01E01, CH01F03a, CH01F03b, CH02A08, CH02A10, CH02B07, CH02C02b, CH02C06, CH02G01, CH02H11, CH03A04, CH04E02 and CH05G07). These primers were selected to provide maximum coverage of the apple genome (i.e. two or three per chromosome), because there was no prior knowledge of the map location of *Pl-d*. Once the linkage group on which *Pl-d* is located was established, additional microsatellites known to map to the same chromosome were tested. These included CH01D03, CH01D09, CH03C02 and CH04D02.

PCRs were performed in 12.5-µl reaction volumes containing 25 ng genomic bulk DNA, 10 mM Tris-HCl (pH 8.0), 1.5 mM $MgCl_2$, 50 mM KCl, 0.25 mM of each dNTP, 0.2 µM forward primer (labelled with [^{33}P] or the fluorescent dyes 6-FAM, NED or HEX, Applied Biosystems), 0.2 µM reverse primer and 0.25 U *Taq* polymerase (Invitrogen). The PCR conditions were as follows: 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 60°C for 45 s and 72°C for 1 min, plus a final extension at 72°C for 6 min. Electrophoresis of the fluorescently labelled products was performed in an automated sequencer (Applied Biosystems 3100) running GENESCAN and GENOTYPER software, whereas the products labelled with [^{33}P]-ATP were separated by electrophoresis on a 6% denaturing acrylamide gel at a constant 65 W for 2–4 h in 1× TBE buffer (Sambrook 1989). The gels were then dried onto Whatman 3 MM paper and the microsatellites visualised following autoradiography (Kodak Biomax MR).

Primer combinations that detected clear differences between the bulks were tested in the ten resistant and ten susceptible individuals comprising the bulks. The remainder of the sub-population and the parents and controls were then analysed with the primers which appeared most tightly linked to *Pl-d*.

RAPD DNA analysis

The same DNA bulks used for the microsatellite and AFLP analyses were also used in a screen of 80 RAPD primers, sets OPA, OPD, OPK and OPL (Operon Technologies). PCR reactions were performed in a 25-µl volume consisting of 50 ng bulked DNA, 10 mM Tris-HCl (pH 8.0), 4 mM $MgCl_2$, 50 mM KCl, 0.125 mM of each dNTP, 0.4 µM single primer and 0.5 U *Taq* polymerase. The PCR conditions were as follows: 94°C for 1 min, followed by 40 cycles of 94°C for 20 s, 35°C for 45 s and 72°C for 2 min, plus a final extension at 72°C for 6 min. Amplified products were separated in 2% agarose gels in 1× TAE (made freshly each time) at 90 V for 2 h. The gels were stained with ethidium bromide and visualised under UV light. Primers revealing clear differences between the bulks were tested in the ten resistant and ten susceptible individuals comprising the bulks. The most tightly linked markers were then used in the whole sub-population.

Mapping

The recombination percentages between the markers and the resistance gene *Pl-d* were first calculated directly from the raw data. The markers showing segregation with mildew resistance were then tested for linkage to *Pl-d* using the program LINKEM (Vowden et al. 1995), which calculated the single-locus segregations and the recombination fractions between the resistance gene (*Pl-d*) and the microsatellite, SCAR, AFLP and RAPD

Table 1 Amplified fragment length polymorphism (*AFLP*) markers for *Pl-d* identified by BSA, cloned and sequenced for conversion to sequence-characterised amplified region (*SCAR*) markers

AFLP primers	Fragment size (bp)	Conversion to SCAR
E-TA + M-CTC	219	Resistant and susceptible sequences identical, conversion to SCAR not achieved
E-AG + M-CAG R1	210	Weak linkage to *Pl-d*, not sequenced
E-AG + M-CAG R2	140	Equivalent to E-AGC + M-CAG R1
E-AG + M-CAG R3	116	Equivalent to E-AGC + M-CAG R2
E-AGC + M-CAG R1	140	SCAR not reliable, inconsistent amplification in susceptible individuals
E-AGC + M-CAG R2	116	Point mutations distinguish R and S sequences, converted to SCAR marker EM DM01

markers. A linkage map was prepared using JoinMap, version 3.0 (van Ooijen and Voorrips 2001).

Marker multiplexing and cultivar survey

A further objective of this work was to produce markers suitable for multiplexing, so that individuals possessing more than one resistance gene could be identified. Ideally they should be screened simultaneously with other markers in the same PCR reaction. PCR conditions were optimised to allow the simultaneous screening of the *Pl-d* SCAR, EM DM01, with the SCAR marker EM M02 for *Pl-w* mildew resistance. The PCR conditions were as described for SSRs above, but with a 53°C annealing temperature, and with 0.2 μM of each EM M02 primer and 0.4 μM of each EM DM01 primer. In addition, to reduce the occurrence of false positives, the SSR markers found to flank *Pl-d* and *Pl-w* were also used to test the progenies. For multiplexing, the SSR primers were combined in equimolar amounts in the same PCR reaction.

The markers were tested in a range of material from the cultivar collection (listed in Table 3) under the optimised PCR conditions described, to establish which genetic background the markers could be multiplexed within. The progeny E711 (*Pl-d* × *Pl-w*) was also tested.

Results

Mildew infection

The results of disease screening over two seasons in the glasshouse identified 103 resistant (grade 0–2) and 129 susceptible (grade 4–5) individuals. Only four individuals scored as grade 3 (intermediate) and 32 individuals died prior to leaf collection; these were not included in the analysis. These data correlated well with the field data, though symptoms were generally less severe in the field. The ratio of 103 resistant to 129 susceptible is reasonably close to the 1:1 ratio expected for a single major resistance gene ($\chi^2 = 2.91$, $P > 0.05$, $df = 1$).

AFLPs and SCAR development

Three sets of AFLP primers of the 176 combinations tested generated six fragments which appeared to be linked to *Pl-d* in a subset of 75 individuals (Table 1). Sequencing confirmed that the two smaller fragments amplified from E-AG + M-CAG corresponded to the two fragments generated by E-AGC + M-CAG. When the SCAR primers designed were tested in resistant and susceptible individuals, only one of the sets of primers (derived from E-AGC + M-CAG) produced a SCAR marker which reliably distinguished resistant and susceptible individuals. The SCAR marker EM DM01 (for: 5′-3′ = AGGATAATAATCTATCTTGTAAAGG, rev: 5′-3′ = CCATTCAGCCCAACGAGT), annealing temperature 53°C, amplifies a product of 90 bp, under the PCR conditions described. Segregation data for the SCAR and closest AFLP, E-TA + M-CTC are shown in Table 2.

Table 2 Segregation of the progeny 'Fiesta' × A871-14 for presence or absence of the alleles coupled with *Pl-d* for the markers SCAR EM DM01, AFLP E-TA/M-CTC and RAPD OPA01 and the microsatellite loci CH01D03, CH03C02, CH01D09 and CH01G12 with resistant (*Pl-d pl-d*) vs susceptible (*pl-d pl-d*) seedlings. Data for CH01D03y, 134/136 bp not presented. χ^2 and recombination values (r) calculated in LINKEM from full genotypic data ($df = 1$, $P < 0.0001$)

Marker/locus	Parent genotypes[a]		+ Marker		− Marker		χ^2	r
	'Fiesta'	A 871-14	Resistant	Susceptible	Resistant	Susceptible		
EM DM01	NA	90	34	2	5	45	59.31	0.08
CH03C02	125/125	125/ 131	33	1	5	46	62.83	0.07
CH01D03z	Null/null	Null/142[b]	4	42	34	5	52.59	0.11
CH01D09	145/152	145/ 149	26	8	12	39	29.35[c]	0.24
CH01G12	108/154	136/149	26	10	12	36	20.83[c]	0.26
E-TA/M-CTC	NA	219	35	5	1	35	54.55	0.08
OPA01	900[b]	NA	3	45	36	2	67.01	0.06

[a] Alleles in coupling with *Pl-d* are *underlined*, *NA* no amplification
[b] Marker in susceptible seedlings scored
[c] $df = 3$, $P < 0.0001$

Microsatellites

The microsatellite primer pair CH01G12 from the first set of microsatellites tested on the bulks showed clear differences between the bulks. This placed *Pl-d* on a linkage group corresponding to group 12 in published maps of apple (Liebhard et al. 2003). Subsequent testing of markers known to map to this group identified three additional markers associated with *Pl-d*, CH01D03, CH01D09 and CH03C02; see Table 2 for details and segregation data in the subpopulation of 86 individuals. For CH01D03, the parental peaks are 134/136 bp ('Fiesta') and 136 bp and 142 bp (A871-14); all individuals in the progeny have the 136-bp peak, while peaks of 134 bp and 142 bp occur together with the 136-bp peak in approximately 22% of the progeny (i.e. 19 individuals have three peaks of 134, 136 and 142 bp). This suggests the presence of a second locus for CH01D03, as reported by Liebhard et al. 2003; CH01D03y (134/136 bp) maps to linkage group 4 in 'Fiesta', while CH01D03z (144 bp) maps to linkage group 12 of 'Discovery'. Because the 136-bp allele occurs in all individuals in the segregating progeny, one parent is presumably 136/136 at locus y, while A871-14 may be 142/null at locus z. The 142-bp allele which segregates with susceptibility was scored and used to infer the map position of the null allele coupled with *Pl-d*. Other markers reported to map to linkage group 12, which would be expected to be close to *Pl-d*, i.e. CH01B12, CH01F02 and CH04D02, did not segregate in the progeny tested. For example, the resistant parent A871-14 ('Worcester Pearmain' × 'D12') was homozygous for CH01B12 (120/120 bp); therefore, resistant and susceptible alleles could not be distinguished.

RAPDs

Four RAPD primers, OPA01, OPK03, OPL01 and OPL13, amplified fragments which appeared linked to *Pl-d*. However, only OPA01 generated a marker which was reproducible in different PCR reactions. The fragment of approximately 900 bp is only amplified in susceptible individuals; the segregation data are summarised in Table 2.

Co-segregation analysis and mapping

The co-segregation data for *Pl-d* and the closest markers—the AFLP, E-TA + M-CTC, the SCAR EM DM01, the RAPD OPA01, and the microsatellites CH01D03, CH01D09, CH01G12 and CH03C02—are summarised in Table 2. The map showing the order of markers is shown in Fig. 1.

The SCAR marker EM DM01 is 9 cM from *Pl-d*, while alleles amplified by the RAPD OPA01 and microsatellite CH01D03z are in repulsion 4 cM and 13 cM, respectively, from *Pl-d*. CH03C02 is 8 cM from *Pl-d*, while CH01D09 is 24 cM from the gene. The position of CH01G12, a further 11 cM from CH01D09, confirms the orientation of the markers on the bottom of a linkage group corresponding to group 12 in published maps.

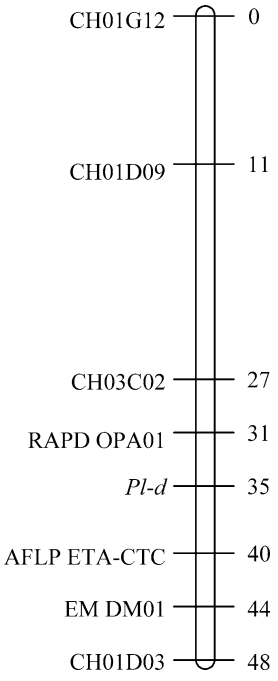

Fig. 1 Linkage map of the *Pl-d* region on linkage group 12, in 'Fiesta' × A871-14, mapped in 86 seedlings, showing the order of markers established in JoinMap. Distances in centiMorgans are indicated on the *right*

Marker multiplexing and cultivar survey

The two microsatellites (CH01D03 and CH03C02) which flank *Pl-d* can be scored simultaneously; thus the probability of correctly identifying individuals with the resistance gene is greater than if a single or non-flanking markers are used. Moreover, the primers can be multiplexed with the microsatellite primers CH05A02 and CH01E12 which are linked to *Pl-w*.

The SCAR marker EM DM01 was successfully multiplexed with the SCAR EM M02 for *Pl-w* (described in Evans and James 2003) in a single PCR which was able to distinguish individuals containing single gene resistances for *Pl-w* and *Pl-d* and identify seedlings which combined both genes in progeny E711 (*Pl-d* × *Pl-w*, Fig. 2).

The closest microsatellite marker, CH03C02, and the SCAR EM DM01 appeared to be robust in the range of genetic backgrounds tested in material from the gene bank at East Malling (Table 3). The SCAR failed to amplify a product in any of the material tested that did not have a background of *Pl-d* resistance, while the CH03C02 primers generated products of a different size to those coupled with *Pl-d*. This will allow *Pl-d* to be screened in the presence of the other mildew resistance genes—*Pl-1*, *Pl-2*, *Pl-w* and *Pl-m*. Moreover, 'Fiesta' and 'Florina' carry genes for resistance to rosy leaf curling aphid (*Dysaphis devecta*) and scab (*Venturia*

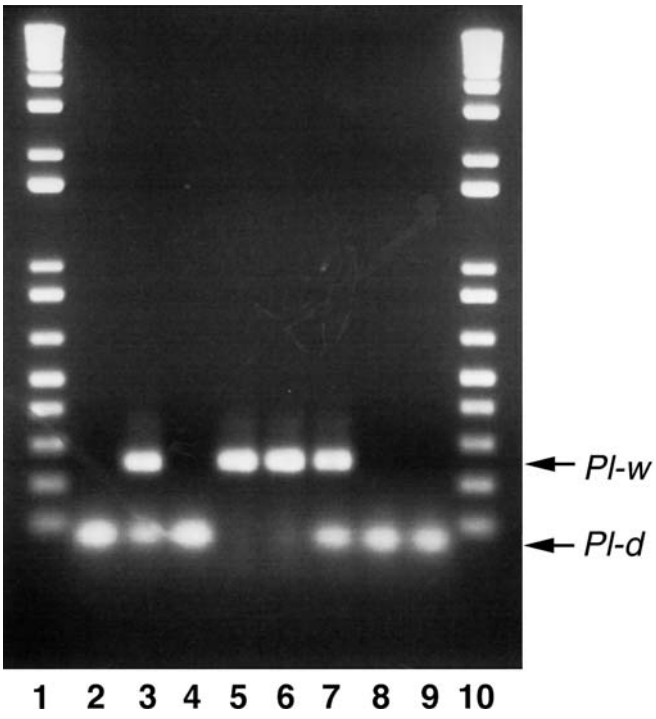

Fig. 2 Markers for *Pl-d* and *Pl-w* amplified from population E711 with SCAR EM DM01 (*Pl-d*) multiplexed with SCAR EM M02 (*Pl-w*). *Lanes 1* and *10* Kb Plus DNA ladder; *lanes 2*, *4*, *8* and *9* have only the *Pl-d* marker; *lanes 5* and *6* have only the *Pl-w* marker; *lanes 3* and *7* have both markers and therefore, these individuals probably have pyramided resistance genes

inaequalis), respectively, and we have found that the *Pl-d* markers can also be used alongside the markers available for these traits (data not presented).

Discussion

We have established the first set of markers linked to the gene for mildew resistance *Pl-d*, using the strategy of BSA followed by segregation analysis. The SCAR marker EM DM01 appears robust in a range of genetic backgrounds and is 'breeder friendly', i.e. can easily be scored on an agarose gel. Furthermore, the forward primer may also be labelled fluorescently for analysis within an automated system if required, thus providing greater flexibility and potential for multiplexing. Two microsatellite markers, CH03C02 and CH01D03, are 7 and 11 recombination units (8 cM and 13 cM), respectively, from the *Pl-d* locus and flank the resistance gene. These markers should prove useful for marker-assisted selection in appropriate breeding lines and further mapping studies. The fact that the markers flank the gene makes them more reliable than a single marker, while the same annealing temperature together with the different sizes of their products makes them amenable to simultaneous testing through multiplexing (though potential difficulties which may arise from the multi-locus marker CH01D03 should be taken into account). A second advantage of the microsatellites is that, unlike the SCAR, AFLP and RAPD markers, they are co-dominant and can therefore be used to distinguish homozygotes and identify individuals which may have two copies of the desired resistance gene. Such individuals could be used as parents in new breeding lines and would pass on the gene to all the progeny in subsequent crosses. The presence of CH01D09 and CH01G12 on the same linkage group confirms the orientation of the markers and, with CH03C02 and CH01D03, should allow the position of *Pl-d* to be inferred in other maps. The position of *Pl-d* on the bottom of linkage group 12 is interesting, because other resistance genes are known to map to this region, for example *Vg* for scab resistance (Durel et al. 2002). It may be that *Pl-d* forms part of a resistance gene cluster and further work is underway to investigate this.

More emphasis is being placed on breeding cultivars with durable resistance by 'pyramiding' major genes to form a multiple-gene barrier against a range of patho-

Table 3 Cultivar survey with the SCAR marker EM DM01 and microsatellites CH01D03 and CH03C02

Accession	Mildew gene	EM DM01[a]	CH01D03 loci y and z[a]	CH03C02[a]
D12	*Pl-d*	90	136/136	125/ 131
A871-14	*Pl-d*	90	136/136 null/142	125/ 131
'White Angel' (1064)	*Pl-w*	NA	135/148	121/125
E295-4	*Pl-w*	NA	135/142	125/127
Mildew immune selection	*Pl-m*	NA	142	123/127
[1]3760 (*Malus robusta* o.p.)	*Pl-1*	NA	135/156	121/125
[1]3762 (*M. robusta* o.p.)	*Pl-1*	NA	135/141	121/133
[1]3752 (*M. zumi* o.p)	*Pl-2*	NA	135	113/121
[1]3753 (*M. zumi* o.p.)	*Pl-2*	NA	135/142	121/125
'Worcester Pearmain'	Polygenic	NA	134/142	113/125
'Fiesta'	Susceptible	NA	134/136	125
'Florina'	Susceptible	NA	138/158	125/127
'Gloster 69'	Susceptible	NA	148/156	125
'Prima'	Susceptible	NA	138/158	125

[a]Alleles coupled with *Pl-d* are *underlined*. The resistance genes previously identified in the accessions are shown in *column 2*. The allele linked to resistance amplified by CH03C02 is not present in the donors of the mildew resistance genes *Pl-1*, *Pl-2*, *Pl-w* and *Pl-m*, or the cultivar with polygenic resistance, 'Worcester Pearmain'. The two loci y and z amplified by CH01D03 could not be distinguished

gens. These new markers for *Pl-d* are not amplified in the sources of the other major mildew genes or polygenic resistance; hence, they can be multiplexed with the markers for these other genes, for example *Pl-w*, and used to identify individuals in a progeny that possess both genes. Moreover, the combination of the *Pl-d* SCAR or microsatellites with the markers currently available for other desirable traits in apple, for example resistance to scab and aphid, should allow rapid high-throughput screening of progenies, thus creating the potential for the development of some exciting new varieties which combine high levels of pest and disease resistance suitable for the expanding organic market.

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References

Alston FH (1983) Progress in transferring mildew (*Podosphaera leucotricha*) resistance from *Malus* species to the cultivated apple. IOBC (WPRS) bulletin: integrated control in orchards. Angers 6:87–95

Butt DJ, Martin KM, Swait AJJ (1983) Apple powdery mildew: damage, loss and economic injury level. In: Proceedings of the 10th international congress of plant protection, Brighton, p118

Dayton DF (1977) Genetic immunity to apple mildew incited by *Podosphaera leucotricha*. HortScience 12:225–226

Dellaporta SL, Wood J, Hicks JB (1983) A plant DNA minipreparation: version II. Plant Mol Biol Rep 1:19–21

Dunemann F, Bräcker G, Markussen T, Roche P (1999) Identification of molecular markers for the major mildew resistance gene Pl_2 in apple. Acta Hort 484:411–416

Durel CE, Lespinasse Y, Calgene F, Van de Weg E, Koller B, Dunemann F, Thiermann M, Evans K, James C, Tartarini S (2002) Four years of the European D.A.R.E. project: numerous results on apple scab and powdery mildew resistance gene mapping. Abstracts of plant and animal genome X, San Diego, p 63

Ellis RP, McNicol JW, Baird E, Booth A, Lawrence P, Thomas B, Powell W (1997) The use of AFLPs to examine genetic relatedness in barley. Mol Breed 3:359–369

Evans KM, James CM (2003) Identification of SCAR markers linked to *Pl-w* mildew resistance in apple. Theor Appl Genet 106:1178–1183

Gallott JC, Lamb RC, Aldwinckle HS (1985) Resistance to powdery mildew from some small fruited *Malus* cultivars. HortScience 20:1085–1087

Gardiner SE, Bus V, Bassett H, Goodman M, Greer L, Ranatunga C, Rikkerink E, Forster R (1999) Identification of molecular markers linked to major resistances to apple scab, powdery mildew and woolly apple aphid in apple. Abstracts of plant and animal genome VII, San Diego

Gardiner SE, Bus V, Rikkerink E, Rusholme R, Meech S, Cook M, Murdoch J, Gleave A, Bassett H, Crowhurst R (2002) Targeted resistance gene mapping in apple using resistance gene analogues from an EST database. Abstracts of plant and animal genome X, San Diego

Gianfranceschi L, Seglias N, Tarchini R, Komjanc M, Gessler C (1998) Simple sequence repeats for the genetic analysis of apple. Theor Appl Genet 96:1069–1076

Guilford P, Prakash S, Zhu JM, Rikkerink E, Gardiner S, Bassett H, Forster R (1997) Microsatellites in *Malus* × *domestica* (apple): abundance, polymorphism and cultivar identification. Theor Appl Genet 94:249–254

Hokanson SC, Szewe-McFadden AK, Lamboy WF, McFerson JR (1998) Microsatellite (SSR) markers reveal genetic identities, genetic diversity and relationships in a *Malus* × *domestica* Borkh. core subset collection. Theor Appl Genet 97:671–683

Knight RL, Alston FH (1968) Sources of field immunity to mildew (*Podosphaera leucotricha*) in apple. Can J Genet Cytol 10:294–298

Korban SS, Dayton DF (1983) Evaluation of *Malus* germplasm for resistance to powdery mildew. HortScience 18:219–220

Krieghoff O (1995) Entwicklung einer In-vitro-Selektionsmethode auf Resistenz von *Malus*-Genotypen gegenüber *Podosphaera leucotricha* (Ell. et Ev.) Salm. und In-vitro-Differenzierung von Virulenzunterschieden des Erregers. PhD Dissertation, Humboldt-Universität zu Berlin

Lespinasse Y (1989) Breeding pome fruit with stable resistance to diseases. 3 Genes, resistance mechanisms, present work and prospects. IOBC (WPRS) bulletin: integrated control of pome fruit diseases. Brissago 1988(12):100–115

Liebhard R, Gianfranceschi L, Koller B, Ryder CD, Tarchini R, Van de Weg E, Gessler C (2002) Development and characterisation of 140 new microsatellites in apple (*Malus* × *domestica* Borkh.) Mol Breed 10:217–241

Liebhard R, Koller B, Gianfranceschi L, Gessler C (2003) Creating a saturated reference map for the apple (*Malus* × *domestica* Borkh.) genome. Theor Appl Genet 106:1497–1508

Markussen T, Kruger J, Schmidt H, Dunemann F (1995) Identification of PCR-based markers linked to the powdery mildew resistance gene Pl_1 from *M. robusta* in cultivated apple. Plant Breed 114:530–534

Michelmore RW, Paran I, Kesseli RV (1991) Identification of markers linked to disease-resistance genes by bulked segregant analysis: a rapid method to detect markers in specific genomic regions by using segregating populations. Proc Natl Acad Sci USA 88:9828–9832

Ooijen JW van, Voorrips RE (2001) JoinMap version 3.0: software for the calculation of genetic maps. Plant Research International, Wageningen

Parlevliet JE (1993) What is durable resistance, a general outline. In: Jacobs TH, Parlevliet JE (eds) Durability of disease resistance. Kluwer, Dordrecht, pp 23–39

Pederson WL, Leath S (1988) Pyramiding major genes for resistance to maintain residual effects. Annu Rev Phytopathol 26:369–378

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning. A laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor

Seglias N, Gessler C (1997) Genetics of apple powdery mildew resistance from *Malus zumi* (*Pl2*). Integrated control of pome fruit diseases. IOBC-WPRS Bulletin 20(9):195–208

Simon CJ, Weeden NF (1991) Elucidation of crab apple lineage by direct examination of rDNA sequences. Malus 5:4–6

Visser T, Verhaegh JJ (1976) Review of tree fruit breeding carried out at the Institute for Horticultural Plant Breeding at Wageningen from 1951–1976. In: Proceedings of the Eucarpia meeting of tree fruit breeding, Wageningen, 1976, pp 113–132

Visser T, Verhaegh JJ (1980) Resistance to powdery mildew (*Podosphaera leucotricha*) of apple seedlings growing under glasshouse and nursery conditions. In: Proceedings of the Eucarpia meeting of fruit tree breeding, Angers, 1979, pp 111–120

Vos P, Hogers R, Bleeker M, Lee TVD, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M (1995) AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res 23:4407–4414

Vowden CJ, Ridout MS, Tobutt KR (1995) LINKEM: a program for genetic linkage analysis. J Hered 86:249–250