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High-resolution genetic analysis of the *Sd*-1 aphid resistance locus in *Malus* spp.

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Abstract Aphids cause serious physical and economic damage to most major crops throughout the world through feeding damage, with consequent symptom development and virus transmission. The rosy leaf-curling aphid (*Dysaphis devecta* Wlk.) is a pest of apple (*Malus* spp.) which displays an exceptionally clear phenotype with respect to susceptible and resistant symptoms. The *Sd*-1 locus for resistance to *D. devecta* biotypes 1 and 2 is present in Cox's Orange Pippin and its progeny and had previously been mapped to the top of linkage group 7. Detailed fine mapping of the locus was initiated with AFLP bulked segregant analysis of both pedigree and segregating bulks, which identified three new marker loci. Preliminary marker order in the *Sd*-1 region was established through mapping in a family derived from Prima × Fiesta, with additional segregation analysis on a Fiesta × Golden Delicious family. Previous recombinant data was re-evaluated and corrected. Two co-segregating AFLP fragments were found to contain a common $(GA)_{23}$ repeat, from which a PCR-based simple sequence repeat (SSR) assay was developed. A high-resolution map around the *Sd*-1 region was established by analysing a large meta-population of *Sd*-1 recombinants using 759 additional individuals from different families. The *Sd*-1 gene has been located within a 1.3-cM interval flanked by the molecular markers SdSSRa and 2B12a and co-locates with the RFLP marker MC064. Allelism between *Sd*-1 and *Sd*-2 resistant sources was tested. Molecular markers tightly linked to *Sd*-1 were shown to be co-segregating with the *Sd*-2 locus, which indicated that *Sd*-1 and *Sd*-2 loci are at least tightly linked and, probably, allelic.

Keywords Aphid resistance gene · Apple · Genetic mapping · *Dysaphis* · *Malus*

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Introduction

Aphids cause serious physical and economic damage to most major crops throughout the world, both directly through feeding damage and symptom development and also because aphid vectors are the primary means of plant virus transmission. As a result, large quantities of insecticides are currently used. These are expensive to apply, cause damage to the ecosystem and environment and are of concern to consumers of fresh produce and processed plant products. The rosy leaf-curling aphid (*Dysaphis devecta* Wlk.) is a pest of cultivated and ornamental apple species (*Malus domestica* and *Malus* spp.). The aphid is monophagous and affects the same trees year after year, causing severe leaf curl with conspicuous red galls. It can cause economic damage to crops of apple in the absence of appropriate control measures (Gratwick 1992).

Resistance to *D. devecta* was first reported by Dicker (1954) who observed that the aphid did not attack the variety Cox's Orange Pippin. Alston and Briggs (1968) showed that resistance in Cox's Orange Pippin, James Grieve, Northern Spy and Ashmead's Kernel was controlled by a single 'gene' or locus. In a later report, Alston and Briggs (1977) described three aphid biotypes and four resistance genes providing resistance to these biotypes. The gene for resistance to biotypes 1 and 2 from Cox's Orange Pippin was given the symbol *Sd*-1, whilst the resistance to biotype 1 only, derived from Northern Spy, was designated *Sd*-2. A further single gene for resistance to biotype 3 was designated *Sd*-3 and derived from *Malus robusta* and *M. zumi*. A number of

deviations from the expected 3:1 or 1:1 segregation ratios were observed in crosses involving varieties containing the *Sd*-1, *Sd*-2 or *Sd*-3 resistance sources (Alston and Briggs 1977). It was therefore proposed that Worcester Permain, Cox, McIntosh, Lane's Prince Albert and MAL68/5 (*M. zumi*, open-pollinated) were heterozygous for a precursor gene, *Pr-Sd*, without which *Sd*-1, *Sd*-2 and *Sd*-3 are ineffective.

To date, there has been one report of a resistance gene from plants being isolated and effective against an aphid (Rossi et al. 1998). The nematode resistance gene *Mi* of tomato also confers resistance against the potato aphid *Macrosiphum euphorbiae*. *Mi* is expressed in leaves, belongs to the nucleotide-binding, leucine-rich repeat family of resistance genes and confers aphid isolate-specific resistance (Milligan et al. 1991; Rossi et al. 1998). It appears that this gene acts by limiting sieve element ingestion (Kaloshian et al. 2000). Other sources of aphid resistance have been described within a range of species, although to date there have been few high-resolution genetic studies reported.

The clear symptom phenotype of the *Malus/D. devecta* system, together with access to a large number of extant segregating families and genetic resources, provides an excellent system with which to characterise and ultimately isolate an aphid recognition resistance gene.

Comprehensive linkage maps for apple cvs Prima and Fiesta have been constructed using DNA and isozyme markers in a full-sib progeny of 152 individuals (Maliepaard et al. 1998). The map and family have been used for genetic analysis of a range of agronomic traits (King 1996; King et al. 1998, 2000; Maliepaard et al. 1998), including resistance to rosy leaf curling aphid (Roche et al. 1997a). The variety Fiesta (*Sd*-1*sd*-1) is derived from a cross of Cox's Orange Pippin (*Sd*-1*sd*-1) × Idared (*sd*-1*sd*-1). The *Sd*-1 gene has been located at the top of linkage group 7, in the context of seven molecular markers [three restriction fragment length polymorphisms (RFLPs) and four random amplified polymorphic DNAs (RAPDs)], with three RFLP markers (MC064a, 2B12a and MC029b) less than 2 cM from *Sd*-1 (Roche et al. 1997a). One of these RFLP markers (2B12a) is closely linked to the *Sd*-1 gene and has been converted to a polymerase chain reaction (PCR)-based marker and used to confirm linkage through pedigree analysis (Roche et al. 1997b).

A successful strategy for map-based cloning of a gene does not depend on any single technique but rather relies on a combination of genetic and molecular biological methods. An important step is the accurate mapping of the target gene based on a large number of recombinant individuals. Once initial linkage is established, a variety of methods can be employed to saturate a target locus with additional DNA markers and thus provide flanking markers which represent a short physical distance, prior to undertaking a chromosome walk. Amplified fragment length polymorphism (AFLP) analysis is one of the most powerful strategies for marker saturation, since it generates large numbers of polymorphic molecular markers in a highly reproducible manner using a minimal amount of DNA (Vos et al. 1995). Bulked segregant analysis (BSA) (Michelmore et al. 1991) is an established method based on bulking DNAs from individual plants that can accelerate identification of polymorphic AFLP fragments linked to the targeted region.

As a prelude to map-based cloning, we have undertaken detailed mapping of the *Sd*-1 aphid resistance region on linkage group 7 of var. Fiesta using AFLP bulked segregant analysis. We have used both family-based and pedigree-based bulks to maximise the chance of detecting tightly linked markers. We describe the fine ordering of markers in the *Sd*-1 region by identification of key recombinants from a large meta-population of additional trees available from a range of mapping and breeding populations involving the resistant apple variety Fiesta. This information was used to select a subset of key recombinants on which accurate scoring of the pest interactions could be carried out as well as the development of reliable co-dominant PCR markers suitable for marker-assisted selection (MAS). In addition, we undertook genetic characterisation of the *Sd*-2 resistance source in order to determine whether there was evidence of allelism between *Sd*-1 and *Sd*-2 rosy leaf curling aphid (biotype 1) resistance sources.

Materials and methods

Mapping population

The mapping family derives from a Prima (*sd*-1*sd*-1) × Fiesta (*Sd*-1*sd*-1) cross (EAGMAP reference family J, Maliepaard et al. 1998) made at CPRO-DLO, Wageningen, the Netherlands in 1988 using cv. Prima as the female parent. Prima is a scab resistant cultivar carrying the *Vf* gene from *Malus floribunda* 821. Fiesta (syn. Red Pippin), derived from a cross between Cox's Orange Pippin and Idared, and contains the gene *Sd*-1 for resistance to two biotypes of rosy leaf curling aphid (*Dysaphis devecta* Wlk.).

Additional Progenies

Leaf material of additional varieties (Table 1) was obtained either from the gene bank collection at HRI, East Malling or the National Top Fruit Collection at Brogdale, Kent.

Following pre-screening with the linked markers, vegetatively propagated clonal scion material was obtained from a number of individuals from families derived from additional crosses involving the resistant variety Fiesta (Table 2). These were bench-grafted onto M9 rootstocks. For allelism tests between *Sd*-1 and *Sd*-2, P-family individuals derived from a cross of Double Red Northern Spy (DRNS) (*Sd*-2*sd*-2) × Totem (syn. SA572/2; *sd*-2*sd*-2) were used. The variety Double Red Northern Spy (DRNS) is a mutant sport of the variety Northern Spy. Additional replicates were located in a field plantation at Wellesbourne grafted onto M27 rootstock (Table 2).

Aphid inocula

In 1999, *Dysaphis devecta* populations were obtained from a private garden in Norwich and from an organic apple orchard in Kent. Both aphids were deduced to be biotype I based on tests with the differential hosts Fiesta and Northern Spy (Alston and Briggs 1997). The aphids overwintered as eggs and in 2000, aphid populations were reared on var. Worcester Permain.

Table 1 Varieties used for the construction of parental resistant (PR) and susceptible (PS) bulks, and their parentage.

Parents		PR Bulk Resistant *Sd*-1	PS Bulk Susceptible *sd*-1	Parents	
Female	Male			Female	Male
Cox	King of the Pippins	Allington Pippin	Laxton's Triumph	Cox	King of the Pippins
Cox	Golden Delicious	Kidd's Orange Red	Ivette	Cox	Golden Delicious
Cox	Wealthy	Fortune	Epicure	Cox	Wealthy
Cox	Cox	642	Elstar	Ingrid Marie	Golden Delicious
Cox	Worcester Permain	Winston	Worcester Permain	Unknown	Unknown
Cox	Worcester Permain	Merton Worcester	–	–	–
Cox	Idared	Fiesta	Idared	Jonathan	Wagener
Prima	Fiesta	J075	J096	Prima	Fiesta
Prima	Fiesta	J006	J002	Prima	Fiesta

Table 2 Segregating families and their parents used to assess aphid symptoms. Determination of reaction to *Dysaphis devecta* in various families during the active growing season at Wellesbourne, with inoculation between May and July and scoring between May and August. A minimum of three to four inoculations were carried out on plants not displaying symptoms

Family	Female parent	Male parent	Number of progeny	Plot	Site[a]	Number of plants screened	Year	Propagation rootstock
A	Fiesta	Starkrimson Red Delicious	176	Field	EM	–	–	M27
			10	Screenhouse	W	10	2000	M9
B	Fiesta	Granny Smith	175	Field	EM	8	1999	M27
			18	Screenhouse	W	18[b]	2000	M9
C	Fiesta	Starkspur Golden Delicious	162	Field	EM	7	1999	M27
			17	Screenhouse	W	17[b]	2000	M9
D	Fiesta	Gloster 69	183	Field	EM	14	1999	M27
			34	Screenhouse	W	34[b]	2000	M9
E	Fiesta	Golden Delicious	63	Screenhouse	W	64	1999	M9
			63	Field	W	–	2000	M27
J	Prima	Fiesta	152	Field	W	85	1999	M27
				Field	W	85[b]	2000	
P	Double Red Northern Spy	SA572/2	133	Field	W	50	1999	M27
				Field	W	50[b]	2000	
642	Cox selfed	–	5	Screen house	W	5	–	M27

[a] EM, East Malling; W, Wellesbourne
[b] Includes 1999 inoculations as well

Assessment of aphid-plant interactions

Aphid screening experiments were conducted either on potted trees in an insect-proof screenhouse or in the field. Inoculations were carried out by placing three to five adult aphids on growing points of the shoots during May, June and July. Inoculated shoots were then covered with perforated transparent bread bags. One shoot on each tree was inoculated in the screen-house, and three different shoots on each tree were inoculated in the field. Symptoms were assessed after 1 week using the scoring procedure of Alston and Briggs (1968). Plants were scored as susceptible (conspicuous galling and reddening of the leaf) or resistant (no symptoms). Those plants without aphid colonies were scored on up to four occasions (Table 2).

Plant DNA extraction

Young leaf tissue was harvested at the active growing stage and refrigerated during transportation to the laboratory prior to storage at –80 °C. Genomic DNA was extracted using a cetyltrimethylammonium bromide (CTAB) miniprep method (Doyle and Doyle 1990). DNA concentration was estimated using a bisbenzimide assay in a Hoeffer 200 DNA fluorimeter and by electrophoresis of genomic DNA samples alongside calf thymus DNA standards on a ethidium bromide-stained 0.8% agarose gel.

Construction of DNA pools

Parental susceptible (PS) and resistant (PR) bulks were constructed based on a previous characterisation of susceptible and resistant varieties (Roche et al. 1997a) (Table 1). An attempt was made to balance the distribution of alleles at loci other than *Sd*-1 between the two bulks. Where possible resistant and susceptible varieties were used which had parents in common. JS and JR bulks were constructed from susceptible and resistant recombinant individuals, respectively selected from the J family (Fig. 1). The recombinant chromosomes in both bulks were derived from the resistant parent, Fiesta.

AFLP analysis

AFLP analysis was performed essentially as described in (Vos et al. 1995) using an AFLP Core Reagent Kit and an Analysis System II Kit (Life Technologies) according to the manufacturer's

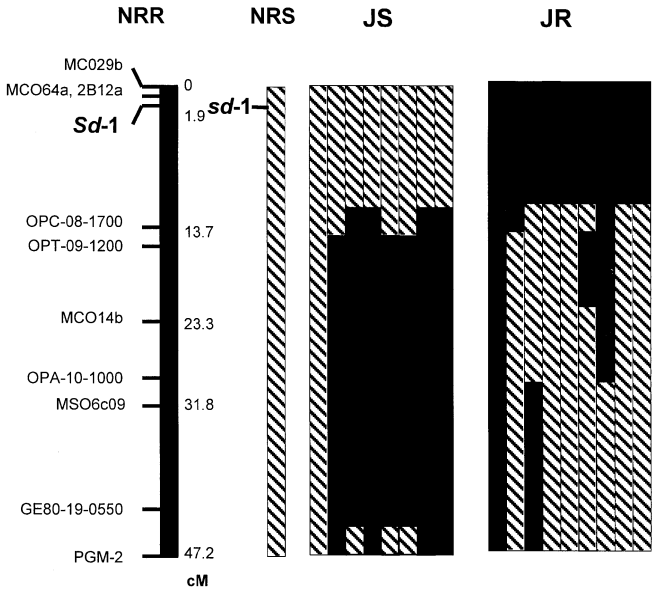

Fig. 1 JS (susceptible) and JR (resistant) bulks were constructed from J family individuals following the identification of recombinant chromosomal segments on Fiesta linkage group 7. *NRR* Non-recombinant resistant, *NRS* non-recombinant susceptible. Recombinant data and original map positions are as presented in (Maliepaard et al. 1998)

instructions. Sixty-four AFLP *Eco*RI/*Mse*I primer combinations using two selective nucleotides at the rare-cutter site (*Eco*RI) and three selective nucleotides at the frequent-cutter site (*Mse*I) were used to screen each of the bulks. Products were separated on 6% denaturing polyacrylamide gels, with electrophoresis at 55 w for 3–4 h. Gels were dried onto Whatman 3 MM paper (without fixing) for 120 min in a vacuum gel drier (Model 583, Bio-Rad) and exposed to film (Fuji RX) at room temperature for 2–4 days.

Cloning and conversion of AFLP markers into PCR-based markers

Fragments corresponding to three AFLP markers (ETC/MCTC-1, ETC/MCTC-2 and ETC/MCTT-1) were excised from dried polyacrylamide gels and eluted prior to re-amplification using the same cycling conditions as for selective PCR. Re-amplified amplicons were ligated into pCRII (Invitrogen) vector. Plasmids containing inserts with the most common sequences were amplified with the original AFLP primers, and those yielding amplicons co-migrating with the AFLP markers were selected for further analysis.

Simple sequence repeat (SSR) analysis

PCR reactions included 4 pmol of each of primers SdSSR-F (GAATTCTCGTCCCTTCATCTC) and SdSSR-R (GTTCCTTAGCCTCCCATTCTG) and were subject to an initial denaturation step at 94 °C for 180 s, followed by 30 cycles of 60 °C for 30 s, 72 °C for 60 s and 94 °C for 30 s, with a final 10-min extension step at 72 °C. The SdSSR-F primer was end-labelled with [33p]-ATP by phosphorylating the 5′ end for fragment detection. The products were separated on a 6% polyacrylamide gel.

DNA sequencing

Cycle sequencing was carried out according to manufacturer's instructions (Perkin Elmer-ABI). BigDye fluorescently labelled dideoxyNTPs were chosen in place of dRhodamine to give extended sequence reads.

Genetic mapping

Linkage analysis was carried out using JoinMap 2.0 (Stam and van Ooijen 1995) with the parameters set for cross-pollinated progeny. All data obtained were checked for the presence of unlikely double recombinants against the existing data sets, and none were identified. Map distances were calculated using the Kosambi mapping function.

Results

Identification of AFLP markers linked to *Sd*-1

In total, more than 7,500 scorable AFLP fragments were inspected for polymorphism on resistant or susceptible bulks. From the 64 different primer combinations tested, five different primer combinations produced six different AFLP fragments that showed polymorphisms between resistant and susceptible pools. Each AFLP candidate marker was re-examined on DNA templates of resistant and susceptible individuals used for bulk construction. This screening resulted in three distinct AFLP markers (named as ETC/MCTC-1, ETC/MCTC-2 and ETC/MCTT-1), obtained with two different primer combinations. These marker fragments were present only in resistant bulk individuals and were therefore in coupling phase with *Sd*-1.

Linkage analysis

AFLP analysis was further extended to 151 J-family individuals and 63 E-family individuals (Fiesta × Golden Delicious; Table 2) to determine the extent of the recombination between the *Sd*-1 locus and the AFLP markers. The analysis of the segregation data suggested that there was no evidence to support any statistically significant departure from a simple 1:1 segregation of the AFLP markers in either the J family (Table 3a) or the E family (Table 3b).

The AFLP markers ETC/MCTC-1 and ETC/MCTC-2 co-segregated with the *Sd*-1 locus and the 2B12a marker in both the J and E families. Two individuals were detected (J004 and E299) which were recombinant between the AFLP marker ETC/MCTT-1 and each of the other adjacent markers, as well as with the *Sd*-1 locus (Table 4a). Using these data, we calculated a preliminary integrated local linkage map around the *Sd*-1 locus at the top of linkage group 7 of var. Fiesta. This positioned *Sd*-1 between the flanking markers MC029b and ETC/MCTT-1, while MC064a, 2B12a and ETC/MCTC-1 and ETC/MCTC-2 co-segregated with the gene.

Re-assessment of existing molecular marker and trait data for Fiesta linkage group 7

The position of the *Sd*-1 gene had previously been determined to be at the top of linkage group 7 of the resistant var. Fiesta. Two RFLP markers (MC064a and 2B12a) were within 1 cM of *Sd*-1 and a third (MC029b) 2 cM

Table 3 Chi-squared analysis of observed segregation ratios of molecular markers and the *Sd*-1 locus amongst the J familiy derived from Prima × Fiesta (**a**) the E family derived from Fiesta × Golden Delicious. (**b**) Expected ratio tested was 1:1, with 1*df*.

a

Locus	Genotypes (♀ × ♂)	Observed segregation aa:ab	χ^2	*P*
2B12a[a]	aa × ab	66:85	2.40	0.122
Sd-1[a]	aa × ab	66:81	1.53	0.210
MC029[a]	aa × ab	65:85	2.67	0.102
MC064[a]	aa × ab	65:84	2.42	0.119
ETC/MCTT-1	aa × ab	66:85	2.40	0.122
ETC/MCTC-1	aa × ab	66:85	2.40	0.122
ETC/MCTC-2	aa × ab	66:85	2.40	0.122

[a]Data from Maliepaard et al. (1998)

b

Locus	Genotypes (♀ × ♂)	Observed segregation aa:ab	χ^2	*P*
2B12a	ab × aa	33:31	0.063	0.80
Sd-1	ab × aa	33:31	0.063	0.80
ETC/MCTT-1	ab × aa	33:31	0.063	0.80
ETC/MCTC-1	ab × aa	33:31	0.063	0.80
ETC/MCTC-2	ab × aa	33:31	0.063	0.80

Table 4 Segregation of *Sd*-1 aphid symptoms and markers in coupling on the Fiesta parental chromosome (*Sd*-1*sd*-1). **a** Segregation of markers and aphid symptoms (*Sd*-1) in key recombinant plants, together with trait scores for J071 and J155, which were corrected from those used in Roche et al. (1997a). For *Sd*-1, ab is resistant and aa susceptible in families A–E, and *vice versa* for family J. **b** Segregation ratios in additional families with Fiesta as female parent

a

Plants	Markers					
	MC029	2B12a	MC064	*Sd*-1	SdSSR	TC/CTT-1
A93	–	ab	ab	ab	aa	aa
A161	–	ab	ab	ab	aa	aa
A164	–	aa	aa	aa	ab	ab
A173	–	ab	aa	aa	aa	aa
A197	–	aa	ab	ab	ab	ab
B165	–	ab	ab	ab	aa	aa
B169	–	ab	ab	ab	aa	aa
C41	–	ab	aa	aa	aa	aa
C46	–	ab	ab	ab	aa	aa
D106	–	aa	ab	ab	bb	ab
D159	–	ab	ab	ab	aa	aa
E299	aa	aa	aa	aa	aa	ab
J004	aa	aa	aa	aa	aa	ab
J068	ab	aa	aa	aa	aa	aa
J071	ab	ab	ab	ab	ab	ab
J155	aa	aa	aa	aa	aa	aa

b

Family	A ab:aa	B ab:aa	C ab:aa	D ab:aa	E ab:aa
SdSSR	89:87	90:85	80:82	138:45	31:32
2B12_V_Fi	90:86	92:83	82:80	95:88	31:32
Sd1	6:4	13:5	8:9	27:7	31:32
MC064	6:4	13:5	8:9	27:7	–
TC/CTT-1	–	–	9:8	–	32:31

from the gene (Roche et al. 1997a). As these molecular and trait data were to be incorporated into the detailed analysis, it was important to confirm that they were reproducible. We therefore re-screened 145 of the J-family individuals with the sequence-characterised amplified region (SCAR) marker 2B12 and 50 individuals with the RFLP markers MC064 and MC029. In 1999, 85 of the J-family individuals, located at HRI Wellesbourne, were also screened with the aphid to test the reproducibility of the trait data obtained previously. During their assessment of the J-family individuals for their reactions to the aphid, Roche et al. (1997a) had identified two J-family individu-

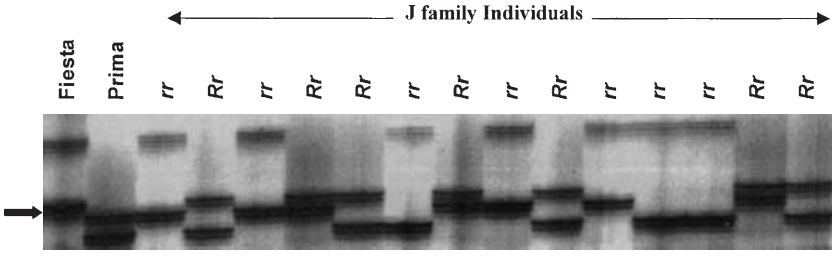

Fig. 2 Autoradiograph showing allele length variation in SdSSR amongst vars. Prima and Fiesta and the segregation of the marker in the J-family individuals. *Arrow* indicates the SdSSRa allele linked to *Sd*-1 in coupling. *Rr* Resistant, *rr* susceptible

als (J071 and J155) which appeared to contain double recombination events between the *Sd*-1 locus and adjacent markers. We were initially unable to reproduce the phenotypic results, which had indicated that J071 was susceptible and J155 was resistant. Repeated inoculation of replicate trees in field and screenhouse assessments during 1999 and 2000 established that J071 was in fact resistant and J155 was susceptible. DNA was extracted from replicate individuals of J071 and J155 from three different locations (Wellesbourne and East Malling in the UK, and Elst in the Netherlands) and the genotype data reanalysed. The marker data were reproducible between trees and confirmed that these individuals did not contain any recombination events between the *Sd*-1 locus and adjacent markers (Table 4a). We therefore amended the trait data records for these particular individuals.

Development of a codominant SSR marker tightly linked to the *Sd*-1 locus

DNA sequence analysis of the AFLP markers ETC/MCTC-1 and ETC/MCTC-2 from cv. Fiesta revealed that these were identical and resulted from the amplification of identical templates containing a $(GA)_{23}$ repeat (EMBL AJ314786). The primers SdSSR-F and SdSSR-R were designed from the flanking sequence, and PCR amplification between these primers resulted in amplicons of 181 bp (SdSSRa) and 203 bp (SdSSRb) in var. Fiesta and of 179 bp and 176 bp in cv. Prima (Fig. 2). Linkage of the marker SdSSRa (the allele which is linked to the *Sd*-1 in coupling) to the AFLP markers ETC/MCTC-1 and ETC/MCTC-2 was confirmed by segregation data obtained from J-family individuals (Fig. 2). No recombinants were observed between the SdSSRa marker and the AFLP markers ETC/MCTC-1 and ETC/MCTC-2.

High resolution location of *Sd*-1

Since no individuals were detected to be recombinant between MC064a, 2B12a, SdSSRa and the *Sd*-1 locus in either of the J or E families, the analysis was extended to a much larger meta-population consisting of additional families segregating for *Sd*-1. Seven hundred and fifty nine additional individuals from different families of A (Fiesta × Starkrimson), B (Fiesta × Granny Smith), C (Fiesta × Starkspur Golden Delicious) and D (Fiesta × Gloster69) were included in the analysis (Tables 2, 4b).

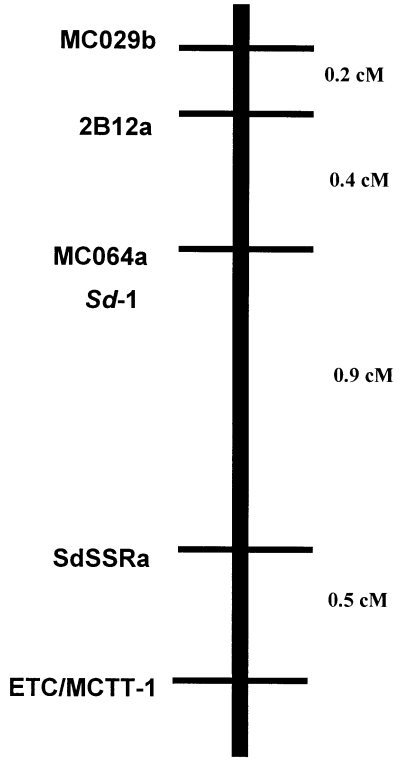

Fig. 3 Fine ordering of the molecular markers and high-resolution integrated linkage map of the *Sd*-1 genomic region calculated from the combined segregation data of six different families. Distances between the markers are given in centiMorgans (cM) using the Kosambi mapping function

The additional families segregating for *Sd*-1 were prescreened with the PCR-based 2B12 (SCAR) and SdSSR markers which had previously been shown to co-segregate with the resistance gene. A 1:1 segregation was observed with 2B12 for all of the families screened. 1:1 segregation ratios were also observed with SdSSRa in three families (Table 4b), with the exception that a 3:1 segregation was observed for D family (Fiesta *Sd*-1*sd*-1 × Gloster69 *Sd*-1*sd*-1). There has been no previous published account of the resistance status of Gloster69. We established in replicated experiments on at least four branches of each of four trees in both 1999 and 2000 that var. Gloster69 is resistant to the aphid and possesses dentical SdSSR alleles as Fiesta. However, it possesses a null allele for the 2B12 SCAR marker.

Eleven plants were identified with recombination break-points between the markers 2B12(SCAR) and

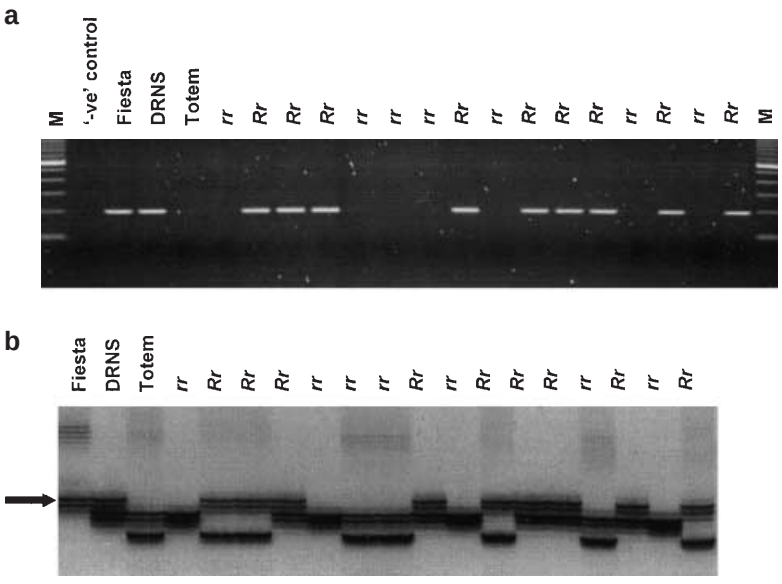

Fig. 4a, b 2B12 SCAR (**a**) and SdSSR (**b**) analysis of vars. Double Red Northern Spy and Totem and the progeny (P family) obtained from the cross between these two varieties. **a** 196-bp 2B12 SCAR amplicon was obtained from vars. Fiesta (*Sd*-1*sd*-1) and Double Red Northern Spy (*Sd*-2*Sd*-2) but no amplification was observed from var. Totem. *M* 100-bp ladder, *Rr* resistant, *rr*, susceptible. *Lane 1* (*M*) Molecular-weight marker, *lane 2* (*-ve control*) PCR reaction without template. **b** The SdSSRa allele, which is tightly linked to the *Sd*-1 in coupling phase is also present in var. Double Red Northern Spy and shows linkage with the *Sd*-2 resistance source in coupling phase. *Arrow* indicates the position of the SdSSRa allele

SdSSR. Of these, four had recombination between 2B12 and *Sd*-1, and seven were recombinant between SdSSR and *Sd*-1. Five of these plants were from the A, two from B, two from C and two from D families (Table 4a). This information was used to select individuals for which aphid assessments were carried out in an insect-proof screen-house. There was evidence of recombination between 2B12 and *Sd*-1 in 4 of the 11 individuals and evidence of a recombinant between SdSSRa and the resistance locus in seven individuals.

The combined data set from the six segregating families was used to generate an integrated linkage map around *Sd*-1 (Fig. 3). *Sd*-1 was located between two flanking markers 2B12a and SdSSRa, which are separated from each other by 1.3 cM. No recombination was observed between MC064a and the *Sd*-1 resistance locus for the 11 plants which had recombination break-points between 2B12(SCAR) and SdSSR.

The *Sd*-2 resistance source co-locates with *Sd*-1

The *Sd*-2 resistance locus had previously been identified as being distinct from the *Sd*-1 locus (Alston and Briggs 1977). We analysed P family individuals derived from a cross of Double Red Northern Spy (*Sd*-2*sd*-2) × Totem (*sd*-2*sd*-2). The individuals were assessed for their interactions with the aphid in the field both in 1999 and 2000. Of the 47 plants tested, 22 were susceptible (*sd*-2*sd*-2) and 25 resistant (*Sd*-2*sd*-2). This conformed with a 1:1 segregation (χ^2=0.66). These individuals were analysed with the *Sd*-1-linked PCR markers 2B12 and SdSSR. A 196-bp amplicon was obtained with the 2B12 SCAR primer pair from resistant var. DRNS, but there was no amplification observed from the susceptible parent, Totem. The progeny was then scored for the presence (ab) or absence (aa) of the 196-bp amplicon (Fig. 4a). The segregation data did not show any deviation from a 1:1 ratio (χ^2=0.66). SdSSR analysis of DRNS revealed the presence of the SdSSRa allele in this variety. This allele is tightly linked to the *Sd*-1 locus in coupling and had been shown to be present in all the individuals containing the *Sd*-1 resistance source. The P-family individuals analysed with marker SdSSR were then scored for the presence or the absence of the SdSSRa allele (Fig. 4b). The segregation data obtained from these individuals did not depart from a 1:1 ratio (χ^2=0.66).

Linkage analysis demonstrated that 2B12a and SdSSRa co-segregated with the *Sd*-2 locus (LOD 15.32), which suggests that both *Sd*-1 and *Sd*-2 loci are located in the same genomic region in *Malus* and raises the possibility that these two resistance sources are allelic.

Discussion

We have successfully determined a reliable map position for the *Sd*-1 aphid resistance gene, within a 1.3-cM interval at the top of *Malus* linkage group 7, flanked by the co-dominant molecular markers SdSSRa and 2B12a. The approach we adopted maximised information from existing segregating families, breeding material and varieties in order to increase the probability of detecting recombination and thus provide a reliable estimate of tight linkage in coupling with the resistant phenotype.

The initial search for additional tightly linked AFLP markers was facilitated by the use of both pedigree and segregating bulks, which provided complementary information on recombinants arising from a wide range of different male and female meioses. Pedigree bulks have been used in the past in conjunction with RAPD markers in *Malus* (Yang et al. 1997). Following the screening of more than 7,500 fragments, only two informative AFLP loci were identified from different primer combinations.

At one of these loci we detected and characterised an AFLP allele which was comprised of two fragments.

The accurate mapping of the target gene and identification of key recombinants in a segregating family is an essential step for map-based cloning. Our preliminary marker order in the *Sd*-1 region was established through mapping in a family derived from Prima × Fiesta, with additional segregation analysis in a Fiesta × Golden Delicious family. We have re-evaluated and corrected previous recombinant data for the Prima × Fiesta family obtained by-Roche et al. (1997a) and corrected two inconsistencies. Previously, 2B12 and MC064a had been placed 1 cM from the *Sd*-1 resistance locus. We re-screened specific individuals and evaluated additional segregating individuals with both the aphid and molecular markers. This enabled us to obtain a stable and more accurate locus order. In this preliminary map we were thus able to establish that markers 2B12a and MC064a co-segregated with the *Sd*-1 locus, while MC029b was located 0.7 cM away from the gene.

Subsequently, a high-resolution map around the *Sd*-1 region was established by extending the analysis to a larger meta-population consisting of an additional four families derived from crosses involving resistant var. Fiesta as the female parent. By analysing *Sd*-1 recombinants, we were able to establish a reliable marker order around the *Sd*-1 region. This places the *Sd*-1 gene at the same recombinant interval as the RFLP marker MC064, within a 1.3-cM interval flanked by the molecular markers SdSSRa and 2B12a. As a result we identified 11 individuals recombinant between the flanking markers. Any linkage map calculated from such segregation data can present only one of several possible marker orders. However, consideration of alternative marker orders would not be consistent with the pattern of segregation presented in Table 4a, and the map presented (Fig. 3) appears to be that most consistent with the data obtained. Confirmation of marker order awaits alignment of the genetic map with any future physical map based upon long genomic clones.

This analysis was facilitated by the availability of individuals from different families derived from crosses involving var. Fiesta. The pre-screening of individuals with PCR-based molecular markers to identify key individual recombinants in the vicinity of *Sd*-1 greatly reduced the labour-intensive aphid screening. This approach is applicable to a wide range of traits where the assessment of phenotype is problematic or costly. The pre-screening of large populations in other plant species with molecular markers flanking the gene of interest has also been successful in identifying informative recombinants for high-resolution map construction (Ballvora 1996; Kaloshian et al. 1998). In the current study, recombinants between markers 2B12 and SdSSR were identified only in crosses involving var. Fiesta as the female parent. A similar difference was also observed in other plant species (DeVicente and Tanksley 1991; Mestre et al. 1997). However, in the present study there were 759 plants for which Fiesta was the female parent, whereas there were only 152 where Fiesta was the male parent. The ability of male and the female meioses to detect the informative recombinants around *Sd*-1 was found not to be significantly different (P=0.05).

No evidence has arisen in this study to indicate that the presence of a precursor gene is required for resistance in any of the families tested. The J and E families did not deviate from the expected 1:1 ratio, which was also consistent with segregation in the A, B and C families. Likewise, the D family did not deviate from the expected 3:1 ratio. There remains a need for larger families to be tested in order to verify the existence of precursor genes in the sources proposed by Alston and Briggs (1977). The use of markers developed in the current study would facilitate the selection of individuals to be tested. Specifically, identification of individuals which carried marker alleles linked in coupling and flanking *Sd*-1, but which did not display a resistant phenotype, would verify the presence of a precursor gene.

Three *D. devecta* biotypes and three different sources providing resistance against these biotypes had previously been suggested (Alston and Briggs 1977). We have shown that var. Double Red Northern Spy, nominally the source of *Sd*-2 resistance, contains the two molecular marker alleles that are linked in coupling and flank the *Sd*-1 locus. In addition, we demonstrated tight linkage between these markers and aphid symptoms in siblings derived from the cross of Double Red Northern Spy × Totem. As a result we conclude that *Sd*-1 and *Sd*-2 are linked in the *Malus* genome, which raises the possibility that the two resistant sources are allelic or components of a complex locus. The ability to verify the distinctness of *Sd*-1 from other sources of resistance is dependent upon the availability of aphid biotypes which display a differential response. Since the only two sources of aphid available to us were consistent with biotype 1, we were unable to test this further. Until such time as biotype-2 or -3 aphids are identified and re-isolated it is not possible to address this issue. In such a situation, verification of allelism of *Sd*-1 and *Sd*-2 would require analysis of progeny in which both sources segregate.

Although AFLP analysis is a powerful technique, the markers are predominantly dominant, costly and technologically demanding. The conversion of AFLP markers to simple PCR-based markers broadens the utility of markers for use in genotype screening and progeny selection. This strategy has been used recently in carrot and pepper (Bradeen and Simon 1998). We were fortunate to identify a simple sequence repeat assay, based on a $(GA)_{23}$ repeat within the AFLP markers E-TC/M-CTC-1 and E-TC/M-CTC-2. The use of the SdSSR marker in combination with 2B12 thus provides practical markers which flank the *Sd*-1 locus (Fig. 3). We also conclude that the SCAR assay developed previously (Roche et al. 1997b), based on 2B12, is closer to the resistance locus than originally published. Molecular markers have also been successfully used in tagging and mapping major genes and quantitative trait loci for plant resistance to insects (reviewed in Yencho et al. 2000).

Host-plant resistance is a method of choice for plant pest suppression. Traditionally, the introgression of desirable genes for pest resistance into an elite cultivar has been carried out via crosses between donor and recipient genotypes, followed by phenotypic selection. The early and accurate selection of individuals carrying functional resistance can improve the efficiency of crop improvement. Isolating and characterising aphid resistance genes may assist in the search for additional sources of resistance and lead to an understanding of the mode of action. In addition, the isolated genes may be deployed via genetic modification into existing elite varieties. The presence of markers closely linked to the gene of interest, together with the availability of large segregating families and large insert genomic libraries allows development of map-based cloning approaches. The accurate characterisation of the *Sd*-1 system, with the clear symptomology defined in *Malus*, is an excellent candidate for such an approach.

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