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K. M. Evans · C. M. James

Identification of SCAR markers linked to *Pl-w* mildew resistance in apple

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Abstract Resistance to powdery mildew is an important objective for cultivar improvement programmes of apple and several different major genes for resistance to mildew are available. Molecular markers linked to such key traits can be used to screen progenies for resistant individuals. A progeny derived from the crab apple 'White Angel' (the source of *Pl-w*) was screened for resistance to mildew for two seasons in the glasshouse and four seasons in the field. DNA bulks of resistant and susceptible seedlings were screened with 176 AFLP primer combinations. Seven AFLP markers were identified that differentiated the bulks, and two of these markers were developed into SCARs, EM M01 and EM M02, mapping at 4.6 and 6.4 recombination units from *Pl-w*.

Keywords Apple · Resistance · Powdery mildew · Molecular marker · SCAR

Introduction

Due to the high levels of infection of powdery mildew (*Podosphaera leucotricha*) in UK apple (*Malus pumila* Mill.) orchards, where growers may apply up to 17 fungicide sprays each year (Butt et al. 1983), resistance to mildew is an important objective of UK apple breeders. Some traditional cultivars and recent releases from breeding programmes have improved resistance to mildew; these tend to carry polygenic resistance that is insufficient to allow significant relaxation of spray regimes. The major genes for resistance to mildew, *Pl-1* from *Malus robusta* (Knight and Alston 1968), *Pl-2* from *Malus zumi* (Knight and Alston 1968), *Pl-w* from 'White Angel' (Gallott et al. 1985; Simon and Weeden 1991), *Pl-d* from D12 (Visser and Verhaegh 1977) and *Pl-m* from MIS ("Mildew immune selection") (Dayton 1977), offer significantly improved levels of resistance when incorporated into cultivars (Knight and Alston 1968).

Combining these resistance genes with good fruit quality characteristics has taken several generations of breeding because the donor species of these genes have small, poor quality, crab apple-type fruit. At HRI East Malling, a range of breeding lines has been developed that carry each of the major genes for resistance to mildew that have been identified.

However, the vulnerability of cultivars carrying only one resistance gene was highlighted recently with the detection of race 6 of *Venturia inaequalis* (scab) that can break down the previously unconquered resistance of the *Vf* gene (Parisi et al. 1993). Also, physiological races of mildew have been isolated that are able to overcome the resistance from *M. robusta* and from *M. zumi* (Krieghoff 1995), and the breakdown of resistance from 'White Angel' and MIS has been reported (Korban and Dayton 1983; Lespinasse 1989).

Many other crops show similar breakdown of monogenic resistance to disease (Parlevliet 1993). The durability of a major resistance gene is limited by the probable development of a virulent race of pathogen. The more widely cultivars with monogenic resistance are cultivated, the more likely this is to happen. By combining major resistance genes to form a multiple gene barrier ("pyramiding"), the chances of the pathogen developing a race capable of breaking down the resistances are reduced greatly. A breeding line, the resistance of which depends on accumulated partial effects of numerous resistance genes, should exert little selection pressure on the pathogen (Pedersen and Leath 1988). However, in the absence of a collection of differential races of mildew, distinguishing these genotypes has so far proved to be impossible.

Some effort has been made to combine major gene resistance with polygenic resistance. It is possible to select individuals combining polygenic resistance with the *Pl-1* gene, due to the necrotic resistance reaction that is spe-

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K.M. Evans (✉) · C.M. James
Horticulture Research International, East Malling,
Kent ME19 6BJ, UK
e-mail: kate.evans@hri.ac.uk
Tel.: +44-1732-843833, Fax: +44-1732-521557

cific to this major gene (Alston 1983), but the other resistance genes provide no such "clues" to aid selection.

The identification of DNA markers for mildew resistances – *Pl-1* (Markussen et al. 1995), *Pl-2* (Seglias and Gessler 1997; Dunemann et al. 1999; Gardiner et al. 1999) and *Pl-m* (Gardiner et al. 2002) – offer breeders the opportunity to select seedlings that carry more than one resistance gene.

The primary target of this work was to identify a marker for an additional resistance gene that can be used alongside those for *Pl-1* and *Pl-2*, to aid in the production of cultivars with more durable resistance. The gene for resistance to mildew is from 'White Angel' (*Pl-w*), an ornamental crab apple from North America related to *Malus sargenti* and *Malus sieboldii* (Simon and Weeden 1991). 'White Angel' has been shown to provide a higher level of resistance than *Pl-1* and *Pl-2*, particularly in glasshouse tests (Alston 1983). *Pl-w* is known to be linked to the isozymes *Acp-3* and *Aat-p* (Hemmat et al. 1994) and *Lap-2* (Manganaris 1989; Batlle and Alston 1996). Further work (James et al., submitted) has shown that *Pl-w* is linked to two microsatellites (Liebhard et al. 2002) that map with *Lap-2* to linkage group 8 of the 'Prima' × 'Fiesta' map reported by Maliepaard et al. (1999).

Materials and methods

Plant material

An apple progeny of 267 seedlings, heterozygous for *Pl-w*, was produced in 1995 by the controlled crossing of 'Fiesta' × E295-4 ('Gloster 69' × 'White Angel'). A subgroup of 85 individuals (plus the parents of the progeny) was used for the analysis of AFLP markers coupled with *Pl-w*.

Disease assessment

The progeny was subjected to natural mildew infection in the glasshouse for two seasons. Symptoms were scored using the scale shown in Table 1. In 1998, the seedlings were planted as cordon rows in an unsprayed plot where mildew inoculum levels were sufficient to screen further for resistance. Primary and secondary mildew symptoms were scored over four seasons using the scales shown in Table 1. Individuals were given a single final score indicating their highest susceptibility score of the ten assessments.

Individuals for bulking were selected from the extreme ends of the scale, 0 (no symptoms) for resistant and 4 or 5 (severe or complete infection) for susceptible; each bulk contained ten individuals. A further 65 individuals, representing the full range of glasshouse and field scores, were selected to check the co-segregation with resistance of the promising AFLP markers identified by bulked segregant analysis.

DNA extraction and AFLP analysis

Young leaves were frozen in liquid nitrogen and stored at –80 °C. DNA was extracted from 1 g of tissue following the SDS/potassium acetate method described by Dellaporta et al. (1983). The DNA was quantified against lambda standards on agarose gels stained with ethidium bromide. The DNA was then diluted to 100 ng/µl and bulks consisting of the ten resistant and ten susceptible individuals were made.

The DNA bulks were screened with 128 primer combinations from the large and small genome AFLP kits (Gibco-BRL Life Technologies) following the manufacturers' instructions, except that the reaction volumes were reduced to 25% of the recommended volumes. The bulks were also digested with the restriction enzyme combinations *Pst*I/*Mse*I and *Kpn*I/*Mse*I, and screened with a further 48 primer combinations using the adapter sequences described in Ellis et al. (1997) and Vos et al. (1995), following the Gibco-BRL protocol. The following selective bases were used with *Kpn*I, K/AA, K/AC and K/AG; while P/ACA, P/ACC and P/ACT were used with *Pst*I. Selective amplification was performed with eight sets of *Mse*I primers (M/CAA, M/CAC, M/CAG, M/CAT, M/CTA, M/CTC, M/CTG and M/CTT), combined with the *Eco*RI, *Pst*I or *Kpn*I primers labelled with γ^{33}P-ATP (Amersham). An equal volume of 80% formamide containing 0.5% bromophenol blue and xylene cyanol was added to the PCR products. The products were denatured at 90 °C for 3 min and separated on a 6% denaturing sequencing gel in 1 × TBE buffer (Sambrook et al. 1989). The gel was dried onto Whatman 3MM paper and the AFLP bands visualised following autoradiography (Kodak Biomax MR).

Primers that detected qualitative differences between the bulks were then tested in 20 resistant and 20 susceptible individuals, and then the remainder of the sub-population if the marker appeared tightly linked.

Isolation and cloning of AFLP fragments

AFLP bands linked to *Pl-w* were excised from the dried polyacrylamide gel with a sterile scalpel and incubated in 50-µl TE (10 mM Tris, 1 mM EDTA pH 8.0) overnight at 4 °C. The fragments were re-amplified in 12.5-µl reaction volumes containing 1.25-µl of template DNA under the conditions described for pre-amplification by the manufacturer. One microliter of PCR product

Table 1 Scales for the evaluation of powdery mildew resistance in the glasshouse and the field

Glasshouse		Field – primary		Field – secondary	
Class	Description	Class	Description	Class	Description
0	No symptoms	0	No symptoms	0	No visible symptoms
1	Very slightly infected	1	Very few clusters infected	1	Very slight infection on leaves
2	Slightly infected	2	Few clusters infected	2	Slight infection on leaves, occasionally on shoots
3	Moderately infected	3	Moderate number of clusters infected	3	Moderate infection on leaves and/or shoots
4	Severely infected	4	Many clusters infected	4	Heavy infection on leaves and/or shoots
5	Completely infected	5	Most clusters infected	5	Very high infection on leaves, most shoots damaged

was diluted 1:30 and used as a template in a second PCR, in a reaction volume of 100 µl containing 150 ng of each appropriate selective primer, 10 mM Tris–HCl pH 8.0, 1.5 mM of $MgCl_2$, 50 mM of KCl, 0.25 mM of each dNTP and 2.5 units of *Taq* polymerase (Gibco-BRL). The touchdown PCR protocol was used as described by Gibco-BRL in the AFLP protocol. The products were separated by electrophoresis through a 1.2% agarose gel in 1 × TAE. Following staining in ethidium bromide and visualisation under UV light, the bands were excised from the gel and the PCR products isolated using the Qiaex II gel-extraction kit (Qiagen) following the manufacturers' instructions.

The isolated fragments were A-tailed and cloned into the bacterial plasmid pGEM-T Easy Vector (Promega) following the manufacturers' instructions. The plasmids were then transformed into the bacterial strain *Escherichia coli* XL 1-blue MRF′ following the method of Pope and Kent (1996), and cultured overnight with standard ampicillin selection and blue/white screening. Plasmid DNA was extracted from positive colonies using the QIAprep spin miniprep kit (Qiagen) following the manufacturers' instructions.

To ensure the correct bands had been cloned, the plasmid DNA was amplified using the appropriate AFLP primers and run adjacent to the original AFLP reactions on a polyacrylamide gel. Plasmids containing sequences of the correct size were sequenced using pUC/M13 primers.

Conversion of AFLP sequences to sequence-characterised amplified region (SCAR)

Primers were designed using the software Primer 3 (Center for Genome Research, Whitehead Institute, Mass.) and DNAStar Primer Select (DNAStar, Madison, Wis.), and commercially synthesised (Sigma-Genosys). The primers were tested in ten resistant and ten susceptible individuals. PCR amplifications were performed in 12.5-µl reaction volumes containing 25 ng of genomic DNA, 10 mM of Tris–HCl pH 8.0, 1.5 mM of $MgCl_2$, 50 mM of KCl, 0.25 mM of each dNTP, 0.2 µM of forward primer, 0.2 µM of reverse primer and 1 unit of *Taq* polymerase (Gibco-BRL). The following conditions were used: an initial denaturation step at 94 °C for 2 min 30 s, followed by 35 cycles of 94 °C for 30 s, annealing temperature for 45 s, 72 °C for 60 s and a final extension at 72 °C for 10 min. The PCR products were electrophoresed through a 1.2% agarose gel in 1 × TAE and photographed under UV light following staining with ethidium bromide. Primers that distinguished resistant from susceptible individuals were then tested in the remaining individuals of the subgroup.

Following the low success rate in converting AFLPs into sequence-characterised amplified regions from the sequences obtained, bands amplified in susceptible individuals by the most tightly linked markers were also cloned and sequenced according to the method described above. The sequences derived from resistant and susceptible individuals were then aligned using the program Megalign within DNAStar, and where possible the primer pairs were re-designed to exploit differences between the resistant and susceptible sequences. These primers were tested in the subgroup of 85 plus a further 29 individuals, making a total of 114.

Mapping

The program 'LINKEM' (Vowden et al. 1995) was used to test the single-locus segregations and estimate the recombination fractions between the SCARs EM M01 and EM M02, the two microsatellites [CH01E12 and CH05A02y (Liebhard et al. 2002)] and *Pl-w*.

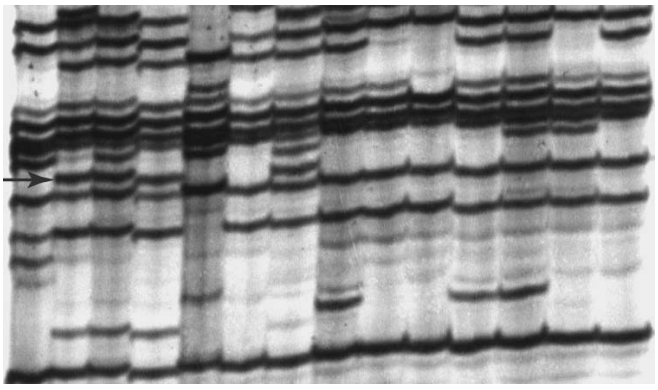

Fig. 1 AFLP banding pattern produced by the primer combination E-ACT + M-CAT in the 'Fiesta' × E295-4 population segregating for mildew resistance *Pl-w*. *Arrow* shows marker linked to *Pl-w* in individuals from the population

Results

Mildew infection

Glasshouse data from the first year show the segregation of the full progeny for mildew resistance as 131 susceptible: 133 resistant. This ratio does not differ significantly from the 1:1 ratio expected with the segregation of a single major gene ($\chi^2 = 0.015$, $P > 0.05$, $df = 1$). Seedlings were classed as resistant when scored as either 0 or 1; scores of 2 or 3 were classed as intermediate and scores of 4 or 5 were classed as susceptible.

The data from the field agreed very well with those from the glasshouse. As expected, mildew symptoms were generally much less severe in the field as the trees matured with scores dropping from, for example, 5 in the glasshouse to 4 in the field. Only two seedlings that had appeared to be resistant in the glasshouse (both scoring 1) were scored as non-resistant (intermediate 3) in the field, but on only one occasion out of the total eight field evaluations. Seventeen of the original seedlings (six resistant and 11 susceptible) died during the evaluation. Of the 250 seedlings left, 118 were resistant (with field and glasshouse maximum scores of 0 or 1) and 122 susceptible (with field and glasshouse maximum scores of 4 or 5), leaving ten seedlings regarded as intermediate (maximum score of 2 or 3). The segregation of the field scores 118:122 did not differ significantly from the expected 1:1 ratio ($\chi^2 = 0.066$, $P > 0.05$, $df = 1$).

AFLPs and SCARs

Seven AFLPs that appeared to be tightly linked to *Pl-w* were identified from the original bulks and tested on the subgroup of 85 individuals. One of these AFLPs is shown in Fig. 1. The AFLPs were cloned and sequenced for conversion into SCARs (Table 2). Two of these (E-ACT + M-CAT and P-ACT + M-CTC) were success-

Table 2 AFLP markers for *Pl-w* cloned and sequenced for conversion to SCARs

AFLP primer combination	Fragment size (bp)	Conversion to SCAR
P-ACT + M-CTC	274	Primers amplify product from R individuals only
E-ACT + M-CAT	129	Primers amplify product from R individuals only
K-AA + M-CAC	172	R product 2 bp smaller than S, only distinguishable on polyacrylamide gel
E-AAG + M-CAG	208	Primers distinguish R from S above 63 °C, but amplification not consistent
E-ACC + M-CAA	174	No amplification from either R or S
P-ACT + M-CAA	136	Do not distinguish R and S
K-AA + M-CTC	149	Do not distinguish R and S

Table 3 Segregation of the progeny 'Fiesta' × E295-4 into mildew resistance classes and for presence or absence of the SCAR markers

Classification of mildew symptoms	EM M01		EM M02	
	Number of seedlings with marker	Number of seedlings without marker	Number of seedlings with marker	Number of seedlings without marker
Resistant (classes 0 & 1)	37	3	37	3
Intermediate (classes 2 & 3)	3	2	4	1
Susceptible (classes 4 & 5)	2	67	8	61

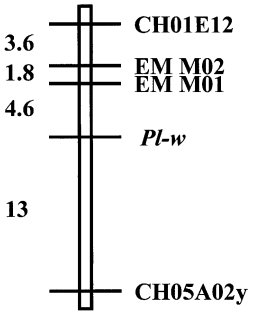

Fig. 2 Map of a section of linkage group 8 showing recombination distances between EM M01 and EM M02, together with the nearest microsatellites (James et al. 2003) and *Pl-w*

fully converted into the SCAR markers EM M01 (F 5′-3′ = GAATTCACTTGTGCTAAG; R 5′-3′ = GGAAAGAAAGACCAAAATAAACG; annealing temperature 50 °C) and EM M02 (F 5′-3′ = CTGCAGACTGTTTGTAAGTTGG; R 5′-3′ = AACTCCTTGATTTCTCCTATTGTT; annealing temperature 56 °C), respectively, that amplify a PCR product only from resistant individuals. The bands were 88 bp and 250 bp respectively. A third set of primers, designed for the AFLP detected by K-AA + M-CAC, amplifies a PCR product which is 2-bp shorter in resistant than susceptible individuals. This difference is detectable only on a sequencing gel. The remaining four SCAR markers did not identify resistant individuals consistently.

Mapping

EM M01 and EM M02 were tested on 114 individuals from the 'Fiesta' × E295-4 progeny; the results, together with mildew scores, are shown in Table 3. The resistant and susceptible seedlings, but not the intermediates, were used to calculate the mapping distances. EM M01 and EM M02 were 4.6 and 6.4 recombination units from *Pl-w*, respectively (Fig. 2).

Discussion

We identified two SCAR markers (EM M01 and EM M02) linked in coupling to the *Pl-w* mildew resistance gene. Although *Pl-w* had been mapped previously to linkage group 8 of the 'Prima' × 'Fiesta' map, it is not realistic to use the isoenzymes *Acp-3*, *Aat-p* and *Lap-2* for high-throughput screening, and the reported microsatellites (CH01E12 and CH05A02y) map 10 and 13 cM from *Pl-w*. These two new SCAR markers (with 4.6 and 6.4 recombination from *Pl-w*) should prove to be more reliable and robust when tested in further progenies.

The maximum mildew score from the glasshouse and field evaluations correlated well with the presence or absence of the markers. Thirty-seven out of the 40 resistant individuals had the EM M01 marker, which was present in only two out of 69 susceptible individuals. The EM M02 marker was also present in 37 out of the 40 resistant individuals; however, there were eight susceptible individuals out of 69 that amplified the marker. Of the five individuals that were classified as intermediate, three amplified the EM M01 marker and four amplified the EM M02 marker. It was decided not to include these seedlings in the mapping calculations as they had only one score high enough to class them as intermediate ('2' or '3') out of the ten assessments. These may be misscores, particularly in the field, where the trees were planted quite close together.

The use of *Pl-w* for mildew resistance has been less widespread in breeding programmes than *Pl-1* and *Pl-2*. The mildew data on the 'Fiesta' × E295-4 progeny collected through two seasons in the glasshouse and four seasons in the field (where both primary and secondary symptoms were recorded) show that *Pl-w* produces a good level of resistance even in UK conditions. It segregates in a clear 1:1 ratio and the correlation between glasshouse and field scores is good. There was no evidence of the complementary genes described by Batlle and Alston (1996) in the segregation of this progeny, perhaps because 'Fiesta' was homozygous *RwRw*.

Major gene resistances have been introduced widely into breeding lines and are still of potential value. However, by continuing to develop cultivars with single monogenic resistance, breeders are ultimately jeopardising the durability of these genes. By combining resistance genes, a more durable barrier will be produced that the pathogen is less likely to overcome.

Our preliminary work has shown that EM M01 and EM M02 can be used to screen for *Pl-w* in the presence of both *Pl-1* and *Pl-d*, as only the *Pl-w* band is amplified in each case. Both SCARs, however, amplify a band (88 bp and 250 bp respectively) in *M. zumi* (the source of *Pl-2*) and in some of its derivatives. The bands were amplified in the *Pl-2* selections A143-12 and A143-24 [Jonathan × 3752 (*M. zumi* OP)]. However, three more advanced *Pl-2* selections [including A679-2 (Seglias and Gessler 1997)] did not amplify a band with either EM M01 or EM M02. Clearly, some care will be necessary in selecting the mildew resistance genes (and in some cases the source of those genes) to combine in the breeding programme if marker-assisted selection is to be employed.

Arguably, the most durable form of resistance would be a combination of major genes together with a polygenic background. A quantitative trait locus (QTL) for polygenic field resistance to mildew was identified and located on linkage group 2 of 'Prima' × 'Fiesta' (Dunemann et al. 2000). It is hoped that this work will be continued and that usable QTL markers will be produced in the near future.

The identification of molecular markers linked to resistance to mildew has, for the first time, given breeders the potential to select combinations of resistance genes and to make full use of the wide range of natural donors of resistance. Very little is understood about the possible additive effects of combining resistance genes or about their interaction with the pathogen. The progenies produced at HRI East Malling will provide the opportunity for a much larger project in collaboration with plant pathologists.

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