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ORIGINAL PAPER

Genome mapping of an apple scab, a powdery mildew and a woolly apple aphid resistance gene from open-pollinated Mildew Immune Selection

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Abstract Apple is host to a wide range of pests and diseases, with several of these, such as apple scab, powdery mildew and woolly apple aphid, being major causes of damage in most areas around the world. Resistance breeding is an effective way of controlling pests and diseases, provided that the resistance is durable. As the gene pyramiding strategy for increasing durability requires a sufficient supply of resistance genes with different modes of action, the identification and mapping of new resistance genes is an ongoing process in breeding. In this paper, we describe the mapping of an apple scab, a powdery mildew and a woolly apple aphid gene from progeny of open-pollinated mildew immune selection. The scab resistance gene *Rvi16* was identified in progeny 93.051 G07-098 and mapped to linkage group 3 of apple. The mildew and woolly aphid genes were identified in accession 93.051 G02-054. The woolly aphid resistance gene *Er4* mapped to linkage group 7 to a region close to where previously the genes *Sd1* and *Sd2*, for resistance to the rosy apple leaf-curling aphid, had been mapped. The mildew resistance gene *Pl-m* mapped to the same region on linkage group 11 where *Pl2* had been mapped previously. Flanking markers useful for marker-assisted selection have been identified for each gene.

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Introduction

Apple (*Malus×domestica* Borkh.) is host to a wide range of pests and diseases, many of which are present in all apple-producing regions in the world (Way et al. 1989). The most common diseases that can devastate apple crops if not controlled are apple scab (*Venturia inaequalis*), powdery mildew (*Podosphaera leucotricha*) and fire blight (*Erwinia amylovora*). Extensive germplasm evaluations have shown that many useful sources of resistance are available for the breeding of pest and disease-resistant cultivars as an alternative to pesticides, the use of which is increasingly being disapproved of by consumers.

Resistance breeding has long been applied to apple as a means to control scab and powdery mildew. Since the earliest germplasm evaluations for scab resistance by Aderhold (1902), who recognised the value of resistant cultivars in the absence of effective fungicides, many sources of resistance have been identified. Genetic studies have shown the presence of both major gene and polygenic resistances in apple germplasm and breeders have discussed the merits and disadvantages of each type since resistance breeding commenced (Williams and Kuć 1969). In spite of the range of resistances available to breeders, most breeding programmes have for a long time relied on the *Rvi6* (*Vf*) gene only for the development of new cultivars with scab resistance (Crosby et al. 1992; Laurens 1999). Today, other major gene and polygenic resistances take a more prominent place in most breeding programmes (Gessler et al. 2006). The same applies for mildew, where the *Pl1* and *Pl2*

genes (Alston 1977) have long been used as the main sources of resistance in a number of programmes.

Resistance breeding is an economical option also for insects for which good sources of resistance are available, such as woolly apple aphid (*Eriosoma lanigerum*), a major pest in many Southern Hemisphere countries. Biological control by the parasitic wasp *Aphelinus mali* has shown promise (Shaw and Walker 1996), but its efficacy can be diminished by non-target effects of pesticides (Bradley et al. 1997). Also, the options for chemical control are increasingly restricted, due to a reduction in the number of pesticides available. For controlling the pest underground, breeding of resistant rootstocks is the preferred approach, as biological control is not effective and chemical control is not sustainable. The Malling–Merton rootstocks, which were specifically bred from 'Northern Spy' (Crane 1937) for the Southern Hemisphere countries of the British Commonwealth (Hatton 1937), have proved to be a very effective means to prevent the pest infesting the roots of apple trees.

Even though many sources of resistance to these pests have been identified, the search for new sources of new resistances is ongoing in order to provide a diverse range of resistance genes as a base for the continued development of new cultivars with pyramided resistances. The use of single gene resistances has been shown to be an ineffective approach to achieving long-lasting resistance, as most of the resistance genes used in apple to date have been overcome by the pathogen or pest at some stage, e.g. *Er1* (Giliomee et al. 1968; Rock and Zeiger 1974; Sen Gupta and Miles 1975) and *Er3* (Sandanayaka et al. 2003) for woolly apple aphid resistance, *Rvi2* (*Vh2*) (Shay and Williams 1956; Bus et al. 2005b) and *Rvi6* (Parisi et al. 1993) for scab resistance and *Pl-m* (Lespinasse 1983) and *Pl2* (Caffier and Laurens 2005; Caffier and Parisi 2007) for mildew resistance. However, the seemingly general durability of gene pyramids found in *Malus* germplasm, e.g. scab resistance in Russian apple R12740-7A and *Malus micromalus*, suggests that combining resistance genes is a valid approach to durable resistance, even if they individually condition differential interactions with the pathogen (Bus 2006b). Over the last 10 years, many of these resistance genes have been mapped to the apple genome (Gardiner et al. 2007) to facilitate the application of marker-assisted selection (MAS) in the development of multiple and durably resistant cultivars.

Today, sourcing new resistances is still an important activity of the New Zealand apple-breeding programme. Plant and Food Research maintains an Apple Genetics Population commenced with seed imported from germplasm collections around the world in order to increase the genetic diversity of apple, a crop not native to New Zealand, and to ensure the long-term development of novel apple cultivars (Noiton and Shelbourne 1992). Some of this germplasm has been evaluated to identify new sources of resistance to pests and diseases (Alspach and Bus 1999; Bus et al. 2000a, 2002; Luby et al. 2002). In this paper, we present the mapping of the apple scab *Rvi16*, powdery mildew *Pl-m* and woolly apple aphid *Er4* resistance genes identified in progenies of an open-pollinated (OP) family derived from 'Mildew Immune Selection' (MIS), which itself is an OP selection of 'Delicious' (Dayton 1977). While named for its mildew resistance, there are no reports on its resistance to apple scab and woolly aphid, which could not be determined either since this accession is not present in New Zealand.

Materials and methods

Plant material and phenotyping

Two progeny of the MIS OP family were used to study the genetics of resistance to one pest and two diseases. The genetics of the *Rvi16* scab resistance was studied on three families across two generations, where the first generation family (173 seedlings) was raised from seed of a 'Splendour'×MIS OP 93.051 G07-098 cross made in 1998. A 'fast-breeding' approach was applied to two selections from this family, AK617 and AK653, in order to accelerate flowering (Austin et al. 2006; Volz et al. 2009), and both were crossed in 2000 with 'Scired' to develop second-generation families consisting of 244 and 210 seedlings, respectively. Both 'Splendour' and 'Scired' are susceptible to apple scab. The screening for scab was performed in spring (September–October) by inoculating the seedlings with *V. inaequalis* at the three to five true-leaf stage in the glasshouse under optimal conditions for scab infection (Gardiner et al. 1996). The F1 family was inoculated in 1999 with conidia from a mixture of isolates used in the breeding programme ('breeding mix'), while in 2001, the F2 families were divided into two, when 105 and 71 seedlings of the AK617 and AK653 families, respectively, were inoculated with the breeding mix and the remainder with single-spore isolate J222, an isolate collected in 1996 from a leaf of an unknown cultivar at the Plant and Food Research orchard at Nelson (Bus et al. 2000b, 2005b). In the third week after inoculation, the seedlings were scored into classes using the scale according to Chevalier et al. (1991). Leaf samples for microscopic observations were collected, cleared in a chloral hydrate solution (Bruzzese and Hasan 1983) and mounted in an arabic gum solution (Cunningham 1972). The leaf sections were examined under brightfield conditions, as well as for autofluorescence of the resistance reactions in the interference blue range (excitation filter 450–490 nm, dichroic mirror 505 nm and

barrier filter 515 nm) on a Nikon Optiphot microscope equipped with epi-fluorescence (Nikon, Tokyo, Japan). Digital images were taken with a CoolSnap camera (Coherent Scientific, Adelaide, Australia) and digitally adjusted for contrast in Photoshop version 6.2 (Adobe, San Jose, CA, USA). The glasshouse assessment was followed by field evaluation of the seedlings for scab resistance over 2 years when the trees were in their fourth and fifth leaf (seasons 2004/2005 and 2005/2006, respectively). The seedlings were scored in late spring/early summer on a six-point scale, where 0=no symptoms; 1=occasional lesions on less than 5% of the leaves; 2=lesions on 5–15% of the leaves; 3=lesions on 16–30% of the leaves; 4=lesions on 31–50% of the leaves; to 5=lesions on more than 50% of the leaves.

Accession MIS OP 93.051 G02-054, erroneously identified as MIS OP 93.051 G7-062 by Gardiner et al. (2007), was used to study the genetics of the resistance to woolly apple aphid (*Er4*) and powdery mildew (*Pl-m*). Approximately half of the seedlings from a 'Fuji'×MIS OP 93.051 G02-054 cross made in 1997 were evaluated for resistance to woolly apple aphid (153 seedlings) and the other half for resistance to powdery mildew (176 seedlings). 'Fuji' is susceptible to both powdery mildew and woolly apple aphid. Woolly aphid screening was performed in the 1998/1999 season by repeatedly inoculating the 4-month-old seedlings with infested shoot pieces in the shadehouse (Bus et al. 2008). After about 3 months, the seedlings were scored on a six-point scale according to Bus et al. (2008), where 0=no aphids or galls and 5=large aphid colony and more than 10 galls. The seedlings in the mildew resistance study were screened in the field without fungicide applications, to allow the uninhibited development of disease. The seedlings were scored on a six-point scale, where 0=no symptoms; 1=occasional small to moderate lesions on leaves; 2=large sporulating areas on leaves and/or occasional infected shoot tip; 3=up to 20% of shoot tips infected; 4=20–50% of shoot tips infected; and 5=more than 50% of the shoots and leaves infected in the 2000/2001 and 2001/2002 seasons, when the trees were in their third and fourth leaf, respectively.

DNA isolation, amplification and genetic marker analysis

Genomic DNA was isolated from young immature leaves using the method described by Gardiner et al. (1996). *Rvi16* was mapped in the 'Scired'×AK617 family, while both *Er4* and *Pl-m* were mapped in the divided 'Fuji'×MIS OP 93.051 G02-054 family. Bulked segregant analysis (BSA) using randomly amplified polymorphic DNA (RAPDs) was used to develop markers linked to the three resistance genes. DNA bulks made of phenotypic extremes, two for resistant and two for susceptible individuals, were screened using 240, 200 and 200 Operon primers for *Rvi16*, *Er4* and *Pl-m*, respectively. RAPD band amplification fragments co-segregating with the resistance were converted into sequence characterised amplified region (SCAR) markers as described by Bus et al. (2008). The SCAR markers developed were checked for co-segregation with the phenotype and then mapped when possible in a reference map developed from a 'Malling 9'×'Robusta 5' (M.9×R5) cross (Celton et al. 2009), in order to assign each resistance gene to a linkage group. Then simple sequence repeat (SSR) markers from the corresponding linkage group, as well as the SCAR markers, were screened over each whole segregating population to construct genetic maps.

Markers developed by Plant and Food Research are those beginning with NZms for the SSR markers, and NZsc for the SCAR markers (Table 1), some of which have been reported previously: NZmsCN943818 (LG3), NZmsDR033893 (LG11) and NZmsPal8 (Celton et al. 2009), and NZscAC20, NZscAY17/AB16 and NZscN18 (Gardiner et al. 2003). Other published SSR markers used were CH02d12 (LG11) and CH04e05 (LG7) (Liebhard et al. 2002); NB109a and

Table 1 The primer pairs (F=forward, R=reverse), their polymerase chain reaction (PCR) conditions and linkage group (LG) of not previously reported markers used in this study to map a scab, a powdery mildew and a woolly aphid resistance gene in open-pollinated 'mildew immune selection' accessions

Marker	Primers	PCR conditions	LG
NZmsCV881251	F CTCGGCAATCGGTAAAGCTA R TATGAACAGTGAAACCCTAACCCTA	Td 65-60	3
NZscAJ12	F CAGTTCCCGTATCAGTAGAATGTAT R AGTTCCCGTGGATAGAGAGTG	Ta 60, 1%F	3
NZscAS07	F GACGAGCAGGCATGGAGCA R GACGAGCAGGAACGAAGAGAT	Ta 65	3
NZscA04F3R3	F AATCGGGCTGAAATGACCGAGGCA R AATTGGTAATGAGAGTGGTGTATG	Td 70-60, 1%F	7
NZmsCN905667	F GTTTAGCGGTTTAGGTGCTTGTG R AGGGGTCTTTGTGTTTCTGG	Td 60-55	11
NZscDR033888	F AGATCAAGAAACATGGGATA R TAGCAGCAGAAATACTGGCAGA	Ta 52, 1%F	11

Ta constant annealing temperature, *Td* touchdown programme annealing temperature, *F* formamide added to the reaction mix

Table 2 The phenotypic ratios for three families from two generations segregating for the *Rvi16* gene for apple scab resistance from open-pollinated 'mildew immune selection' (MIS) accession 93.051 G07-098

Female parent	Male parent	Inoculum	Phenotypic class[a]						Total	Segregation[b]		χ^2	*P*
			0	1	2	3A	3B	4		R	S	1:1	
'Splendour'	MIS OP 93.051 G07-098	Mixture	7	42	20	32	16	56	173	101	72	4.86	0.03
'Scired'	AK617[c]	Mixture	7	23	7	28	5	35	105	65	40	5.95	0.01
		J222	59	2	5	1	6	66	139	67	72	0.18	0.67
'Scired'	AK653[c]	Mixture	2	0	6	30	6	27	71	38	33	0.35	0.55
		J222	81	5	0	3	1	49	139	89	50	10.94	0.00

The F2 families were inoculated with two *Venturia inaequalis* inocula: an isolate mixture or single-spore isolate J222

[a] Phenotypic classes according to Chevalier et al. (1991)

[b] R=resistant (classes 0 to 3A); S=susceptible (classes 3B and 4)

[c] The 'Splendour'×MIS open-pollinated 83.051 G07-098 selections AK617 and AK653 exhibited class 1 and class 3A resistance symptoms, respectively

NH030a (LG3) (Yamamoto et al. 2002); EMPc111a (LG7) (Fernández-Fernández et al. 2006); Hi02c06 (LG11), Hi05b09 (LG7), and Hi07d12b (LG7) (Silfverberg-Dilworth et al. 2006); and CH-Sd1 (Khan et al. 2007).

The polymerase chain reaction conditions for the SCAR and SSR markers were carried out as described by Bus et al. (2008). The mapping strategy used was that of the double pseudo testcross (Grattapaglia and Sederoff 1994). Linkage analysis and genetic map construction were performed using JoinMap v3.0 software (http://www.kyazma.nl) with the Kosambi mapping function and the critical logarithm of the odds score for marker grouping set at 5. After an initial mapping round, the genotype data was examined for the presence of double recombinants that possibly corresponded to genotype-phenotype incongruent (GPI) individuals (Gygax et al. 2004). Double recombinants were removed from the analysis to generate the final maps.

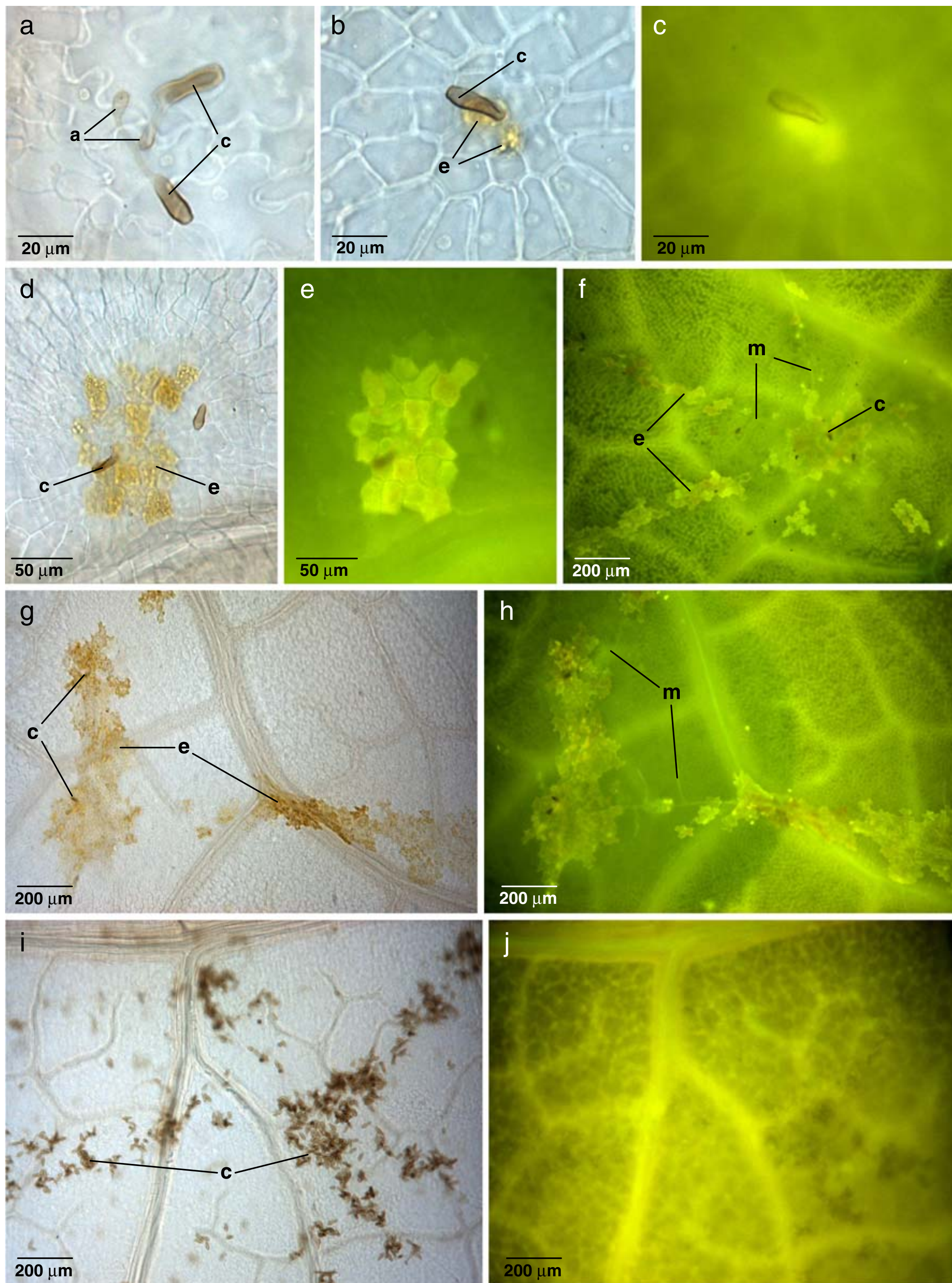
Genetic markers linked to each of the resistance genes were then evaluated on DNA from MIS (Germplasm Resources Information Network (GRIN) accession PI 589818; GMAL 2451) itself to determine the origin of the alleles.

Results

Genetics and symptoms of resistance

The phenotypic data for the families showed strong bimodal segregations for all three resistance genes. The 'Splendour'×MIS OP 93.051 G07-098 family inoculated with the *V. inaequalis* breeding mix in the glasshouse segregated into three main phenotypic groups: hypersensitive response (HR; disease classes 0 and 1), chlorotic resistance reaction (Chl; class 3A) and susceptibility (S; classes 3B and 4), and a minor group: (stellate) necrosis (class 2) (Table 2). The distinct phenotypic resistance classes, combined with resistance to susceptible (R:S) segregation ratios that were skewed towards resistance, suggested the presence of two scab resistance genes in accession MIS OP 93.051 G07-098. Therefore, two progeny from this family, AK617 from the HR class and AK653 from class 3A, were crossed with the same susceptible parent, 'Scired'. However, the F2 families showed very similar segregation ratios depending on the inoculum applied. They segregated into the same three major classes as the F1 family when inoculated with the isolate mixture (Table 2). In contrast, both F2 families inoculated with the single isolate J222 showed very strong bimodal distributions into HR and S classes that did not differ significantly from R:S=1:1 ratios expected from a major gene for the AK617 family, but not for the AK653 family (Table 2). Microscopic observations of the resistance

Fig. 1 Microscopic observations of scab resistance reactions on leaves of 'Scired'×AK617 progeny. **a**–**c** Arrested development of *Venturia inaequalis* isolate J222 on seedling BD228, which was assigned to class 0 (no visible symptoms) in the glasshouse screen. **a** Brightfield observation of germinated conidia that have developed appressoria but have not invoked a resistance reaction in the host. **b**, **c** Brightfield (**b**) and interference blue autofluorescence (**c**) observations of a hypersensitive response (HR) involving two epidermal cells on seedling BD228. **d**, **e** Brightfield (**d**) and interference blue autofluorescence (**e**) observations of an HR involving multiple epidermal cells on seedling BD266 that was inoculated with a mixture of *V. inaequalis* isolates. **f** Interference blue autofluorescence observation of a stellate necrotic (SN) reaction on seedling BD175 inoculated with a mixture of isolates. **g**–**j** Differential interactions of the isolate mixture on seedling BD280, which was assigned to classed 3A according to Chevalier et al. (1991). Brightfield (**g**) and interference blue autofluorescence (**h**) observations of an HR/SN reaction confirm the presence of a resistance gene in BD280. However, the limited sporulation that is visible in the brightfield (**i**) observation is not associated with a resistance reaction in the host as it does not show autofluorescence (**j**). (*a*=appressorium, *c*=conidium, *m*=mycelium, *e*=necrotic epidermis)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symptoms in the glasshouse phenotyping confirmed the high scab resistance conditioned to isolate J222 by the *Rvi16* gene. Many conidia on the class 0 seedlings germinated and produced appressoria but were unable to establish infections (Fig. 1a), while the development of other spores that did achieve infection were rapidly restricted. The HR responses varied from macroscopically invisible single cell (Fig. 1b) to multiple cell (Fig. 1d) necrosis of the epidermis, which involved the production of autofluorescent compounds in the resistance reaction (Fig. 1c, e) that was distinctly independent of the background autofluorescence from the vascular bundles and cell walls of the leaves. The breeding mix induced resistance reactions in the form of stellate necrosis (Fig. 1f) as well as multiple cell HR (Fig. 1g, h), sometimes combined with fungal stroma development showing limited sporulation that was not necessarily associated with autofluorescence of the underlying leaf tissues (Fig. 1i, j). The fungal stroma itself also showed autofluorescence in incompatible interactions as an indication of stress (Fig. 1f, h), but not in what appears to have been a compatible interaction on a class 3A seedling (Fig. 1j). All the resistance reactions were very superficial as they were only expressed in the epidermis and rarely involved the palisade mesophyll (infection on the adaxial side of the leaf) or the spongy mesophyll (infection on the abaxial side of the leaf). The phenotypic scores from the scab screening in the glasshouse were largely confirmed in the field, although a considerable number of the HR seedlings showed low levels of sporulation and a few seedlings could be considered susceptible since they showed higher levels of sporulation (Table 3). However, the correlation between the glasshouse and field scores was low for the seedlings in the intermediate resistance classes 2 to 3B for both the isolate mixture and isolate J222 from the glasshouse screen (Table 3). Therefore, a preliminary map around the scab resistance gene was developed first involving only the HR (classes 0 and 1) and susceptible (class 4) seedlings before the final mapping of the gene based on all seedlings for which marker data were generated.

The major gene nature of both the powdery mildew and woolly apple aphid resistance genes were confirmed in the MIS OP 93.051 G02-054 family. The mildew sub-family segregated into 89 resistant (class 0) and 87 susceptible (classes 1–5) seedlings ($P(\chi^2>0.02)=0.89$) and the woolly aphid sub-family into 80 resistant (class 0) and 69 susceptible (classes 1–5) seedlings ($P(\chi^2>1.47)=0.23$).

Resistance gene mapping

Apple scab resistance gene Rvi16

BSA resulted in the identification of six RAPD markers (OPAJ12/1,400, OPAJ18/500, OPAN01/1,800, OPAS07/700, OPAX6/1,400 and OPAX18/800 bp) linked to apple scab resistance. Two of these (NZscAS07 and NZscAJ12) were successfully converted into SCARs, and a local map around the gene was developed, showing that NZscAJ12 was located closest to the gene. Since NZscAN01 had previously been mapped to linkage group 3 (LG3) of 'Robusta 5' (Peil et al. 2008), *Rvi16* was assumed to be located on this LG. Therefore, SSRs from LG3 were screened over the population and the influence of the inoculum on the phenotypes of the surviving seedlings in the field, which by then was skewed towards resistance as many susceptible seedlings had died, and the mapping of the scab resistance gene was investigated further. We found that a relative large proportion of the seedlings that were susceptible to the breeding mix in the glasshouse actually exhibited the markers NZmsCN943818 and NZscAS07 that had been mapped close to the gene (Table 4). Most of these seedlings indeed were resistant in the field, which suggested that the field evaluation provided a better assessment of scab resistance than the glasshouse screen. An example of this was seedling BD280 (Fig. 1g–j), which was classed 3A in the glasshouse and therefore assumed susceptible in the preliminary assessment (see above) but exhibited both markers, while showing partial resistance in the field. The correlation between the glasshouse screen with *V. inaequalis* isolate J222 and the field screen was much higher than with the breeding mix (Table 4), hence the sub-population of 'Scired'×AK617 screened with J222 was used to map the resistance. Local genetic maps of

Table 3 Comparison of the phenotypic segregations of family 'Scired'×AK617 for scab resistance in the glasshouse by *Venturia inaequalis* inoculum and the field scores

Field score sum	Glasshouse class						Total
	0	1	2	3A	3B	4	
Breeding mix							
0	3	13	1	6	0	1	24
1–3	3	4	3	7	0	0	17
4–6	0	0	1	7	3	11	22
7–9	0	0	2	2	2	9	15
Total	6	17	7	22	5	21	78
Isolate J222							
0	26	1	1	0	0	0	28
1–3	17	1	0	0	3	1	22
4–6	5	0	4	0	2	21	32
7–9	0	0	0	0	0	14	14
Total	48	2	5	0	5	36	96

The phenotypic classes in the glasshouse were assigned in 2001 according to Chevalier et al. (1991), and the field classes were the cumulative disease scores for each seedling from the 2004/2005 and 2005/2006 seasons

Table 4 Comparison of the phenotypic and genotypic segregations of family 'Scired'×AK617 for scab resistance in the glasshouse by *Venturia inaequalis* inoculum and the field scores

Phenotype	Marker	Breeding mix		Isolate 222	
		Glasshouse	Field	Glasshouse	Field
NZmsCN943818					
Resistant	Present	29	50	47	48
Resistant	Absent	1	6	5	5
Susceptible	Present	28	7	4	3
Susceptible	Absent	42	37	44	44
Total		100	100	100	100
Number of seedlings		72		81	
NZscAS07					
Resistant	Present	27	44	55	52
Resistant	Absent	4	9	3	6
Susceptible	Present	27	9	5	8
Susceptible	Absent	42	38	37	34
Total		100	100	100	100
Number of seedlings		64		64	

The percentages and total number of seedlings are presented for markers NZmsCN943818 and NZscAS07. The phenotypic classes in the glasshouse were assigned in 2001 according to Chevalier et al. (1991), and the field classes were the cumulative disease scores for each seedling from the 2004/2005 and 2005/2006 seasons. Classes 0 and 1 from the glasshouse screen were regarded resistant and classes 2 to 4 susceptible, while the seedlings with field score sums of 0 to 3 were classed as resistant and those with a sum of 4 or more as susceptible

Rvi16 based on the glasshouse and field evaluations, respectively, were constructed using four SSRs and two SCARs. As the map positions of each of the markers and the gene did not differ more than 1 cM between both maps, only the map based on the glasshouse phenotyping is presented. *Rvi16* maps at a distance of about 5 cM below NZmsCN943818 towards the lower end of LG3 (Fig. 2). The alleles of this marker and SSR marker NH030a, which maps with NB109a to the same region on LG3 (Celton et al. 2009) just above the gene (Fig. 2), linked to the *Rvi16* resistance gene were amplified with the DNA from the original MIS accession (Table 5).

Woolly apple aphid resistance gene Er4

BSA resulted in the identification of a RAPD band obtained from the OPA04 primer co-segregating with *Er4*. The band was converted into a SCAR marker (NZscA04F3R3), which was mapped to LG7 in the M.9×R5 map. More framework SSRs from this LG were then screened over the whole population, and the position of *Er4* was located between the SSR markers Hi07d12a and CH04e05, at about a third down the linkage group, after seven potential GPIs were removed. The alleles of the markers CH-Sd1 (Khan et al. 2007), which mapped 17.9 cM above the gene (Fig. 2) and CH04e05 linked to *Er4*, were only present in the MIS OP 93.051 G02-054 accession and not in MIS itself (Table 5).

Powdery mildew resistance gene Pl-m

A 1,800-bp product of RAPD marker OPAC20 was found to be distantly (14 cM) linked to *Pl-m*. It was converted into a SCAR marker that mapped close to *Pl-m*. However, it did not segregate in the M.9×R5 reference population. A set of SCARs previously found to be linked to other powdery mildew resistance genes was tested, and five SCARS linked to *Pl2* were also linked to *Pl-m* (Gardiner et al. 2003). As *Pl2* had been assigned to LG11 (Liebhard et al. 2002) with the aid of a marker for a major mildew resistance quantitative trait locus in A679-2 (Seglias and Gessler 1997), SSRs from this group were screened in the MIS population to confirm the linkage. The final genetic map of LG11 was constructed using five SSRs and six SCARs to locate *Pl-m* after seven potential GPIs were removed (Fig. 2). With the gene mapping between NZscAC20 and NZscDR033888 in the upper half of LG11, the linkage of *Pl-m* with *Pl2* was confirmed (Gardiner et al. 2007). The marker allele of CH02d12 linked to *Pl-m* was amplified in both MIS OP 93.051 G02-054 and MIS itself (Table 5).

Discussion

Apple scab resistance gene Rvi16

The new major gene for scab resistance, *Rvi16*, which previously was reported under its 'working name' *Vmis* (Gardiner et al. 2007), was mapped towards the lower end of LG3, at some distance below the region where a scab QTL from 'Fiesta'×'Discovery' was located (Durel et al. 2004). Its independent segregation from other scab resistance genes and its somewhat distinctive resistance symptoms indicate that the gene confers a different resistance mechanism than the other genes and QTLs. The gene also maps below the region where a major QTL for fire blight resistance from R5 was mapped recently (Peil et al. 2007, 2008). Interestingly, a large effect pear scab resistance QTL co-locates with *Rvi16* on LG3 of 'Abbé Fétel' (Pierantoni et al. 2007), suggesting the existence of potential orthologous scab resistance genes in the highly collinear apple and pear genomes (Yamamoto et al. 2004). Also, the linkage of *Rvi16* with marker NH030a suggests that it maps to a region that is homeologous to a region either at the lower end of LG11 or at the top of LG1 through its linkage with marker CH03g12. Neither region is known to carry genes or QTLs for scab resistance.

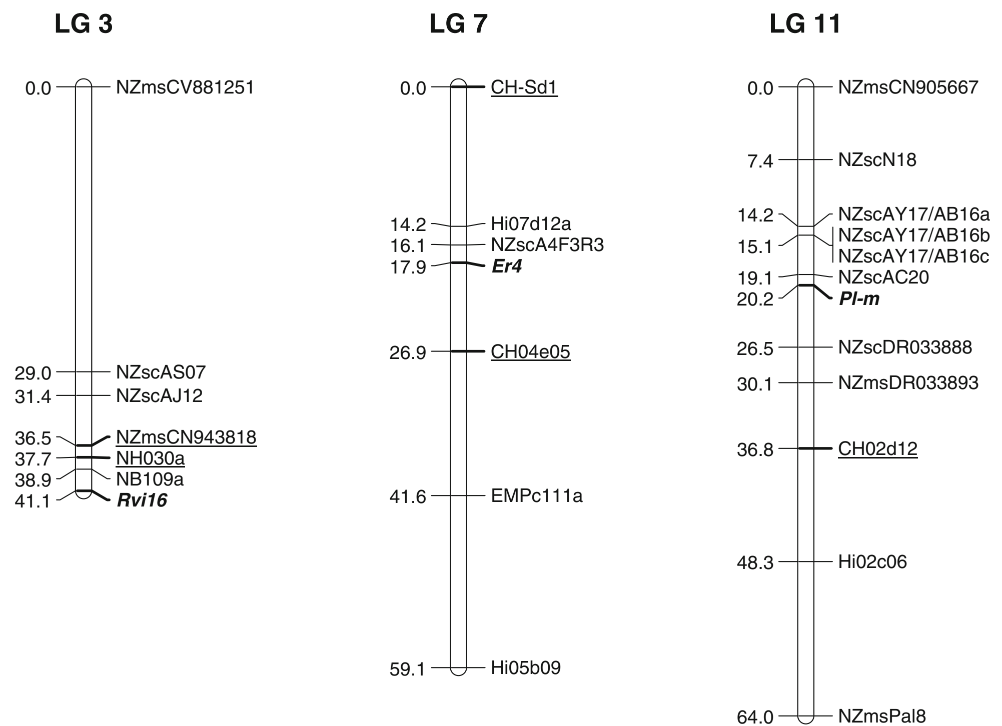

Fig. 2 Genetic maps of the apple scab resistance gene *Rvi16*, the woolly apple aphid resistance gene *Er4* and the powdery mildew resistance gene *Pl-m* identified in accessions derived from open-pollinated 'Mildew Immune Selection'. The *underlined* microsatellite markers were used to trace the origins of the three resistance genes (see Table 5)

While there is a strong suggestion that the gene originated from MIS itself, it is possible that the unknown pollen parent may have contributed the gene, and further research will be required to resolve this question. *Rvi16* is named according to the recently proposed nomenclature system for gene-for-gene relationships in the *V. inaequalis*–*Malus* pathosystem, based on the fact that it segregates independently of all the other scab resistance genes named to date (Bus et al. 2009).

While the gene conditions an HR in the host to certain isolates, such as J222 in our experiment, the gene conditions partial resistance under different disease pressures and/or environmental conditions involving different pathotypes of the pathogen. This was best demonstrated by the very similar segregation ratios of the F2 families derived from accessions AK617 and AK653, in spite of these resistant seedlings having been classed as 1 and 3A, respectively, after inoculation of the 'Splendour'×MIS OP 93.051 G07-098 family with the breeding mix. Some seedlings also sustained low levels of infection in the orchard, but the field screening, together with the glasshouse screening with a single isolate, strongly suggested that only one major gene is involved in the scab resistance of MIS OP and allowed the gene to be mapped more

Table 5 Genetic comparison of 'mildew immune selection' (MIS) with accessions carrying the apple scab resistance gene *Rvi16* (AK617 derived from open-pollinated (o.p.) MIS 93.051 G07-098), the powdery mildew resistance gene *Pl-m* and the woolly apple aphid resistance gene *Er4* (o.p. MIS 93.051 G02-054), the susceptible parents and two reference cultivars

Genetic marker	Accession						
	MIS	AK617	'Scired'	G02-054	'Fuji'	'Fiesta'	'Discovery'
Rvi16							
NH030a	202, *210*	202, *210*	202, 212			200, 202	200, 202
NZmsCN943818	*198*, 216	*198*, 216	206, 216			216	216
Er4							
CH-Sd1	239, 253			239, *261*	243, 253	237, 245	249, 263
CH04e05	217, 227			*211*, 217	197, 225		
Pl-m							
CH02d12	197, *205*			*205*, 223	211, 219	201, 219	219

The alleles are not corrected to their true size, and those printed in italic are linked to the relevant resistance gene

precisely. Previous findings that multiple assessments of accessions carrying the *Rvi6* scab resistance gene in the glasshouse and the field increased the reliability of linkage mapping (King et al. 1998) were similar to ours with *Rvi16*. However, the absence of autofluorescence in one of the differential interactions on the leaves of seedling BD280 showing some sporulation strongly suggests that a race exists in the New Zealand *V. inaequalis* population that can overcome the gene. Further studies will be performed to investigate this.

The microscopic observations of the resistance reactions showed that they were very superficial as they only affected the epidermis of the leaves. Also, fewer cells were involved in the resistance reactions compared to the HR conditioned by the *Rvi5* (*Vm*) and *Rvi4* (*Vh4*), and the SN conditioned by the *Rvi2* (*Vh2*) and *Rvi8* (*Vh8*) scab resistance genes, which usually involve the palisade mesophyll (Win et al. 2003; Bus et al. 2005a; Bus 2006a). The resistance reactions on hosts carrying the *Rvi16* gene therefore have a much more subtle structure and are less intrusive on the leaves than the reactions conditioned by the other genes.

Woolly apple aphid resistance gene *Er4*

The woolly apple aphid resistance gene *Er4* from accession MIS OP 93.051 02-054 whose 'working name' was *Er-m* (Gardiner et al. 2007) mapped to LG7. It segregates independently from the three woolly aphid resistance genes that were recently mapped to LG8 and LG17 (Bus et al. 2008) and therefore is expected to confer a different mechanism of resistance than these genes. The name *Er4* follows on from the naming of the *Er1*, *Er2* and *Er3* genes. Since the marker alleles linked to the gene in MIS o.p. 93.051 G02-054 of neither CH-Sd1 nor CH04e05 flanking the gene were shared with MIS itself, the gene appears to be derived from an unknown pollen parent. The *Er4* gene maps to the upper region of LG7, about 21 cM below the linked *Sd-1* and *Sd-2* genes for resistance to the rosy leaf-curling aphid (*Dysaphis devecta*; Cevik and King 2002a, b), which originally were identified in 'Cox's Orange Pippin' and 'Northern Spy', respectively (Alston and Briggs 1968, 1977). The genes therefore map too far apart to be part of the same resistance gene cluster and to have similar functionalities. The only other resistances mapped to LG7 to date are a minor QTL for leaf scab resistance (Liebhard et al. 2003) and a QTL for fire blight resistance from 'Fiesta' (Calenge et al. 2005; Khan et al. 2007). Both are located well below *Er4* towards the bottom end of this linkage group.

Powdery mildew resistance gene *Pl-m*

The *Pl-m* gene for mildew resistance present in MIS OP 93.051 G02-54 originated, as expected, from MIS and was mapped to LG11. As its name indicates, MIS was originally recognised as a potential source for mildew resistance in apple breeding but has not been used much since the accession was found to be infected by mildew in 1978 (Korban and Dayton 1983). In a glasshouse experiment, it was shown that MIS was strongly infected by mildew inoculum from Illinois and to a lesser extent by inoculum from Pennsylvania, but not other inocula (Lespinasse 1983). The differential interactions strongly suggested that a race of *P. leucotricha* had developed; however, MIS was not infected in the field in the years following from 1978 (Korban and Dayton 1983). As there are no later reports of the *Pl-m* gene having been overcome, the gene still has value for resistance breeding, provided it is pyramided with other mildew resistance genes to achieve durable resistance. While it was demonstrated previously that the *Pl-m* and *Pl2* genes are linked (Gardiner et al. 2003) and might be allelic, they however are different genes or alleles as hosts carrying the *Pl-m* gene are resistant to a *P. leucotricha* race overcoming the *Pl2* gene in New Zealand (Wood and Bus, unpublished data). The gene maps to the same region of LG11 where a scab QTL identified in 'Fiesta' has been repeatedly mapped (Durel et al. 2003, 2004; Liebhard et al. 2003) as well as one from 'Gala' (Soufflet-Freslon et al. 2008). Although a number of QTLs for powdery mildew resistance have been identified in 'Discovery'×TN10-8 family, none of them mapped to LG11 (Calenge and Durel 2006).

Conclusion

In this paper, we have described the identification and mapping of an apple scab, a powdery mildew and a woolly apple aphid resistance gene in two OP derivatives of MIS. The genes will be very useful in the development of new apple cultivars with gene pyramids for multiple, durable resistances. Back-cross lines have been developed to introgress these genes into new apple cultivars with pyramided resistances, with the aid of MAS using the (flanking) markers identified in this study.

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