Tree Genetics & Genomes (2008) 4:233–236
DOI 10.1007/s11295-007-0103-3

ORIGINAL PAPER

Genome mapping of three major resistance genes to woolly apple aphid (*Eriosoma lanigerum* Hausm.)

V. G. M. Bus · D. Chagné · H. C. M. Bassett · D. Bowatte · F. Calenge · J.-M. Celton · C.-E. Durel · M. T. Malone · A. Patocchi · A. C. Ranatunga · E. H. A. Rikkerink · D. S. Tustin · J. Zhou · S. E. Gardiner

Received: 9 January 2007 / Revised: 9 April 2007 / Accepted: 29 May 2007 / Published online: 25 July 2007

Abstract Woolly apple aphid (WAA; *Eriosoma lanigerum* Hausm.) can be a major economic problem to apple growers in most parts of the world, and resistance breeding provides a sustainable means to control this pest. We report molecular markers for three genes conferring WAA resistance and placing them on two linkage groups (LG) on the genetic map of apple. The *Er1* and *Er2* genes derived from 'Northern Spy' and 'Robusta 5,' respectively, are the two major genes that breeders have used to date to improve the resistance of apple rootstocks to this pest. The gene *Er3*, from 'Aotea 1' (an accession classified as *Malus sieboldii*), is a new major gene for WAA resistance. Genetic markers linked to the *Er1* and *Er3* genes were identified by screening random amplification of polymorphic deoxyribonucleic acid (DNA; RAPD) markers across DNA bulks from resistant and susceptible plants from populations segregating for these genes. The closest RAPD markers were converted into sequence-characterized amplified region markers and the genome location of these two genes was assigned to LG 08 by aligning the maps around the genes with a reference map of 'Discovery' using microsatellite markers. The *Er2* gene was located on LG 17 of 'Robusta 5' using a genetic map developed in a M.9×'Robusta 5' progeny. Markers for each of the genes were validated for their usefulness for marker-assisted selection in separate populations. The potential use of the genetic markers for these genes in the breeding of apple cultivars with durable resistance to WAA is discussed.

Keywords *Malus* · *Eriosoma lanigerum* · *Er* · Resistance gene · Genetic map · Genetic markers · Woolly apple aphid

Communicated by A. Abbott

V. G. M. Bus (✉) · M. T. Malone · A. C. Ranatunga · D. S. Tustin
The Horticulture and Food Research Institute of New Zealand Ltd,
HortResearch Hawke's Bay, Private Bag 1401, Havelock North 4157, New Zealand
e-mail: vbus@hortresearch.co.nz

D. Chagné · H. C. M. Bassett · D. Bowatte · J.-M. Celton · S. E. Gardiner
HortResearch Palmerston North,
Private Bag 11 030, Palmerston North 4442, New Zealand

E. H. A. Rikkerink
HortResearch Mt Albert,
Private Bag 92169, Auckland 1142, New Zealand

J. Zhou
School of Resources Management, Southwest Forestry College,
Kunming, People's Republic of China

F. Calenge · C.-E. Durel
UMR 'GenHort' Génétique et Horticulture,
INRA Centre d'Angers,
49071 Beaucouzé cedex, France

A. Patocchi
Plant Pathology, Institute of Integrative Biology,
ETH Zürich,
8092 Zürich, Switzerland

Present address:
A. Patocchi
Agroscope Changins–Wädenswil Research Station ACW,
Plant Protection, Phytopathology,
P.O. Box 185, Schloss,
CH-8820 Wädenswil, Switzerland

Introduction

Woolly apple aphid (WAA; *Eriosoma lanigerum* Hausm.) is a widespread pest of apples that is particularly critical to

the economics of the apple industry in Southern Hemisphere countries such as New Zealand. WAA infestation of the tree and/or root system reduces shoot growth and hence production capacity (Brown and Schmitt 1990; Brown et al. 1995) and in extreme cases will lead to tree death. WAA infestations on mature fruit also reduces market access to several Asian countries. Although biological and chemical control measures are applied to keep infestations on the above ground parts of the tree in check, WAA infestations have increased recently, as more susceptible cultivars, such as 'Braeburn,' 'Fuji' and 'Cripps Pink,' have been planted by growers. At the same time, the number of effective WAA insecticides available to growers has decreased (Lemoine and Huberdeau 1999) because they no longer meet legislative requirements. Rootstocks with genetic resistance that usually are field immune to the pest are used to prevent infestation of the belowground parts. Until recently, most scion cultivars in New Zealand were grafted on either MM.106 or M.793 rootstocks, both of which were derived from the resistant cultivar 'Northern Spy' in the 1920s and 1930s (Crane et al. 1936). These rootstocks were specifically developed in response to the devastating effect WAA had on the burgeoning British Empire Southern Hemisphere apple industries in the 1920s. However, in recent years, growers have increasingly been planting apple trees on dwarfing M.9 and M.26 rootstocks, which are very WAA susceptible, to increase the economic efficiency of production. This may be a major step backwards (Wilton 1998) resulting in unsustainable practices to control the pest, such as insecticide drenching of the rootzone, being required. Hence, breeding for resistance to WAA is an important objective for New Zealand scion and rootstock-breeding programmes.

A number of extensive germplasm evaluations for sources of WAA resistance have been performed (e.g. Cummins et al. 1981; Jancke 1937; Speyer 1924) as a first step to resistance breeding, but reports on the genetics of resistance have been scarce to date. Crane et al. 1936 investigated the genetics of WAA resistance in 'Northern Spy' as part of the Malling–Merton rootstock breeding programme, but it was not until about 30 years later that re-interpretation of their data led to the naming of the major gene *Er* in 'Northern Spy' (Knight et al. 1962). The *Er* gene was renamed *Er1* with the identification of another major WAA resistance gene, *Er2* (King et al. 1991; Alston et al. 2000). However, the assignment of *Er2* to accession 3762 by King et al. (1991) was erroneous and hence has created confusion about the correct source for this WAA resistance gene. Accession 3762 is *M.×robusta* o.p. accession MAL59/1 raised from seed collected from the Hillier Nurseries (Dunemann et al. 2004). Today, it is regarded as the main source of the *Pl1* gene for powdery mildew resistance (Alston et al. 2000) and has been used in a number of apple-breeding programmes around the world (Dunemann et al. 2004). Two other progeny from *M. robusta*, MAL59/8 and MAL59/9, have also been reported as breeding sources of *Pl1* (Alston 1977; Knight and Alston 1968). There is no information, however, on WAA resistance in any of these *M.×robusta* derivatives, except for accession MAL59/9, today known as accession 3760 (Tobutt, personal communication), having been reported as moderately resistant (Alston et al. 1971) to resistant (Alston 1983) to this pest. In the absence of reports on genetic studies on WAA resistance of this accession, the association of the *Er2* gene with *M.×robusta* MAL59 therefore is spurious. However, another source of *M.×robusta*, 'Robusta 5', had been reported highly resistant (Cummins et al. 1981; Mackenzie and Cummins 1982), but no genetic studies were performed to assign a name to its WAA resistance gene. This source was selected after it was shown to be immune to 'Spy-capable' WAA (Young et al. 1982) and has been used extensively in the New York apple rootstock breeding programme for the development of WAA-resistant rootstocks (Cummins and Aldwinckle 1983). We therefore propose that 'Robusta 5' is assigned as the progenitor of *Er2* WAA resistance from the species *M.×robusta*.

A putative new gene for WAA resistance, *Er3*, was identified in *Malus sieboldii* 'Aotea 1' by Bus et al. (2002). This accession was selected as a rootstock for its resistance to root canker (*Peniophora sacrata*) and WAA from an accession (source 32) of open-pollinated seed received from the Morioka Horticultural Research Station, Japan (Taylor 1981). It had originally been introduced into New Zealand as *Malus prunifolia* but was later identified as *M. sieboldii* based on its morphological characters (Yan, personal communication). The species had not previously been noted as possessing WAA resistance, as the evaluation of three other accessions of *M. sieboldii* had shown that they were moderately to highly susceptible (Cummins et al. 1981; Young et al. 1982).

The ability of WAA to overcome the *Er1*-derived resistance (Giliomee et al. 1968; Rock and Zeiger 1974; Sen Gupta and Miles 1975) demonstrated the poor durability of single-gene resistances where race-specific resistance genes are deployed in the absence of a breeding strategy that ensures their durability. There also has been the suggestion of the *Er2* gene having been overcome (Cummins and Aldwinckle 1983), while a biotype of *E. lanigerum* has recently been identified in New Zealand that can overcome the *Er3* gene (Sandanayaka et al. 2003, 2005). Inevitably, races of WAA will spread into or develop *de novo* in other areas where race-specific resistance genes are deployed (Cummins et al. 1981; Knight et al. 1962) in the absence of a breeding strategy to ensure they remain effective in the long term.

To achieve durable resistance to WAA, it is therefore important to not only identify alternative sources of resistance but also to pyramid the resistance genes to effectively develop horizontal resistance. This is best achieved through the application of marker-assisted selection (MAS). MAS through the use of molecular markers closely linked to resistance genes is a powerful selection tool that accelerates the breeding of new more durably resistant cultivars containing two or more pyramided resistance genes. Current phenotyping methods for selection of plants resistant to WAA are cumbersome and cannot distinguish plants containing combinations of resistance genes from those carrying a single gene. While genetic markers have been identified for the *Er1* gene (Bus et al. 2000b), neither the *Er1* nor the *Er2* gene has been mapped on the apple genome to date. A suggestion that the *Er1* locus could be linked to the (self-)incompatibility (SI) locus (Knight et al. 1962) was supported by preliminary research indicating that *Er1* was linked with the isozyme marker *Got-1* for the SI locus (Manganaris and Alston 1987). However, these suggestions were not substantiated in a later study using a small population (50 plants) to investigate the potential linkage of *Er1* resistance with the SI locus with the aid of the *Got-1* and *Got-4* isozyme markers (Tobutt et al. 2000). Preliminary findings on the identification of genetic markers for *Er3* and the application of MAS have been reported previously (Bus et al. 2000b), but the map location on the apple genome has not been reported to date.

In this paper, we confirm the identification of the *Er3* gene from 'Aotea 1' and report the identification of molecular markers for resistances to WAA conferred by the *Er1*, *Er2* and *Er3* genes as well as their map locations. The recent availability of genetic maps that cover the entire apple genome makes it possible to infer the genome location of resistance genes, and the microsatellite markers used provide a valuable resource of highly polymorphic and transferable markers (Liebhard et al. 2002; Silfverberg-Dilworth et al. 2006). Using such markers, partial maps constructed around the *Er1* and *Er3* genes using bulked segregant analysis (BSA) were aligned with a reference genetic map. *Er2* was located on a genetic map constructed in a progeny segregating for the *Er2* gene (Celton et al. 2006). The closest markers to the *Er1* and *Er3* genes were converted from random amplified polymorphic deoxyribonucleic acids (DNAs; RAPDs) to robust sequence-characterized amplified region (SCAR) markers suitable for use in MAS and were validated in double-blind trials. This is the first report where more than two major resistance genes against a single pest or disease have been placed simultaneously onto the genetic map of apple. It illustrates the quickening pace of advancement in locating major resistance genes in this important crop, which is reflected in the different approaches taken to map the genes.

Materials and methods

Plant material and phenotyping for resistance to WAA

The plant material used to confirm the genetics of inheritance of the *Er1* (families 1A–1F), *Er2* (families 2A–2F) and *Er3* (families 3A–3O) genes is described in Table 1. The inheritance of the *Er1* gene was followed over two generations and that of the *Er3* gene over three generations. The progenies were screened either in the glasshouse in the first year after sowing or at a later stage in the field, either as seedlings in their second year in the nursery (planting distance 1.2×0.2 m) or as older seedlings in the orchard (planting distance 3×0.5 m) or as rootstocks in stoolbeds (planting distance 2×0.5 m) at the Hawke's Bay Research Orchard of HortResearch (Table 1). The plants in the glasshouse were raised from seed in PLIX Hillson Rootrainers® baskets (Vertex Pacific, Auckland, NZ) and inoculated with WAA when about 4 months old. All progenies were repeatedly inoculated by placing shoot pieces or seedlings from other crosses with heavily infested root plugs in the canopy of each seedling in the field or in between the young seedlings in the glasshouse. The initial WAA were randomly collected from Hawke's Bay orchards, and colonies were maintained on susceptible plant material at the research orchard. There was no evidence of biotypes being present in this inoculum that could overcome any of the WAA resistance genes being studied. Family 1C was phenotyped twice in the glasshouse in August 2002 (winter) and in March 2003 (autumn). Interference with the WAA population build-up by the parasitic wasp *Aphelinus mali* was prevented by the application of selective insecticides, mostly carbaryl and occasionally cyhalothrin, at regular intervals of about 10–14 days from the start of the inoculations until resistance assessment.

WAA infestation was assessed 3–4 months after inoculation using two six-point scales appropriate to the size of trees. For the young seedlings, the levels of infestation of the root collar and roots were assessed on a scale adapted from Dozier et al. (1971) as described in Table 2A. For the seedlings in the field and the stoolbeds, the scale as described in Table 2B was applied, taking tree size into account. The initial premise for the genetic marker identification was to consider plants classified as 0 to be resistant and those scoring 1–5 to be susceptible. The χ^2 test was applied to the segregations to determine the presence of a major resistance gene.

Bulked segregant analysis with RAPD primers and their conversion to SCAR markers

BSA (Michelmore et al. 1991) was used to identify markers for the *Er1* and *Er3* genes by screening RAPD primers

Table 1 The families used to study the genetics for three woolly apple aphid resistance genes: *Er1* from 'Northern Spy,' *Er2* from 'Robusta 5,' and *Er3* from 'Aotea 1'

Family	Year of cross	Year of screen	Cross		Generation	Screen location
			Female parent	Male parent		
Er1, 'Northern Spy' and derivatives						
1A[a]	1991	1997	'Sciglo'	'Northern Spy'	F1	Orchard
1B	1999	2001	'Northern Spy'	'Braeburn'	F1	Glasshouse
1C[b]	2003	2005	'Northern Spy'	'Royal Gala'	F1	Glasshouse
1D	2003	2005	'Northern Spy'	'Braeburn'	F1	Glasshouse
1E	1997	1999	'Pinkie'	A108R12T161[c]	F2	Glasshouse
1F	1998	2000	X3189	A108R12T146[c]	F2	Glasshouse
Er2, 'Robusta 5'						
2A[a]	1997	1999	X3189	'Robusta 5'	F1	Nursery
2B	1998	2000	'Royal Gala'	'Robusta 5'	F1	Glasshouse
2C	1999	2001	'Braeburn'	'Robusta 5'	F1	Glasshouse
2D	2003	2005	'Royal Gala'	'Robusta 5'	F1	Glasshouse
2E	2003	2005	'Robusta 5'	'Braeburn'	F1	Glasshouse
2F[b]	1997	2006	M.9	'Robusta 5'	F1	Stoolbed
Er3, 'Aotea 1' and derivatives						
3A[a]	1986	1995	M.9	'Aotea 1'	F1	Stoolbed
3B	2002	2004	M.9	'Aotea 1'	F1	Glasshouse
3C	2002	2004	'Aotea 1'	'M.9'	F1	Glasshouse
3D	2002	2004	'Braeburn'	'Aotea 1'	F1	Glasshouse
3E	2002	2004	'Royal Gala'	'Aotea 1'	F1	Glasshouse
3F	2003	2005	'Aotea 1'	'Braeburn'	F1	Glasshouse
3G	2003	2005	'Aotea 1'	'Royal Gala'	F1	Glasshouse
3H[b]	1996	1999	'Royal Gala'	S26R01T053[d]	F2	Nursery
3I	1996	1999	'Royal Gala'	S26R02T008[d]	F2	Glasshouse
3J	1998	2000	'Braeburn'	S26R02T132[d]	F2	Nursery
3K	1999	2001	'Braeburn'	S26R01T053[d]	F2	Glasshouse
3L	2002	2004	'Cox's Orange Pippin'	S26R02T022[d]	F2	Glasshouse
3M	2002	2004	'Braeburn'	S26R02T022[d]	F2	Glasshouse
3N	2002	2004	A45R14T055	A175R01T032[e]	F3	Glasshouse
3O	2002	2004	A45R14T055	A175R01T046[e]	F3	Glasshouse

The progenies were screened either in the glasshouse or in the field as seedlings in the nursery or orchard, or as rootstocks in stoolbeds. The phenotyping scales for the glasshouse and field screens are presented in Table 2.

[a] Family used for marker identification.

[b] Family used for marker validation/marker assisted selection.

[c] A108='Sciglo'×'Northern Spy'.

[d] S26=M.9×'Aotea 1'.

[e] A175='Royal Gala'×S26R01T053.

(Operon Technologies, Alameda, CA) over DNA pooled from phenotypic extremes, i.e. class 0 for resistant and class 5 for susceptible progeny. DNA was isolated from two expanding leaves less than 1.5 cm long that had been harvested from each seedling in spring and frozen at −70°C until use. The extraction protocol was according to Gardiner et al. (1996). Each pool contained DNA from 12 progenies, and DNA bulks of two resistant and two susceptible progeny pools from the *Er1* population (family 1A) were screened using 420 decamer RAPD primers. For the *Er3* population (family 3A), three DNA bulks were screened, one of resistant progeny and two susceptible bulks. DNA screening was performed with 101 single decamer RAPD primers and 50 primer pairs. RAPD reaction mixtures and the amplification program in a Hybaid PCR Express Thermal Cycler (Thermo Electron, Waltham, MA) were as described by Gardiner et al. (1996), except that the annealing temperature was 37°C for the *Er1* screen vs 40°C for *Er3*. Reactions were set up using a Beckman Biomek 2000 Laboratory Automation Workstation (Beckman Coulter, Fullerton, CA), except for the reactions for the *Er3* marker identification screen, which were set up manually. Polymerase chain reaction (PCR) products were separated using 0.9% agarose gel electrophoresis and stained using ethidium bromide. PCR primers giving rise to RAPD products that co-segregated with

Table 2 Assessment scales used for the phenotyping of apple seedlings for woolly apple aphid resistance

Score	Characteristics
A. Seedlings less than 1 year old in the glasshouse	
0	No infestation
1	1–5 aphids/1 gall
2	5–10 aphids/2–3 galls
3	Small colony/3–5 galls
4	2–3 colonies/5–9 galls
5	Large colony/>10 galls
B. Seedlings and stoolbeds in the field	
0	No infestation
1	Light infestation consisting of several small, separate colonies
2	Medium infestation and galling with some colonies starting to coalesce
3	Many colonies coalescing and up to 2 shoots completely infested and galled
4	Heavy infestation and galling on 2-5 shoots
5	Heavy infestation and galling on more than 5 shoots

resistance or susceptibility in the bulks were confirmed across a sub-set of DNA from 22 seedlings and the parents. If the co-segregation was maintained, markers were then screened across DNA from the larger mapping populations. The RAPD markers that mapped were designated with the prefix *NZra_*.

To convert RAPD markers into SCAR markers (Paran and Michelmore 1993), specific DNA fragments amplified from the resistant parent with RAPD primers were excised from the gel and re-amplified twice using the same reaction conditions to increase the amount of the fragment synthesized. DNA was then purified from an agarose gel slice using the Promega Wizard® *Plus* system (Promega, Madison, WI) and cloned into the pGEM-T vector (Promega) using TA cloning. Transformation was into *E. coli* TG1, and the selection for transformants was performed on Luria–Bertani/ampicillin/isopropyl-β-D-thiogalactopyranoside/X-Gal agar plates. White colonies were selected and plasmids purified using the Promega Wizard® *Plus* system. The plasmids were screened for inserts of the expected size by PCR using M13/pUC forward and reverse sequencing primers. Three plasmids were sequenced, and PCR primer pairs (Table 3) were designed using Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). It was necessary to construct a second forward primer (OPO05-OPF02) for screening the *Er3* validation population (family 3H) because of lack of polymorphism between the parents with the first set of primers. The PCR reaction mix for the SCAR markers was the same as for the RAPDs, and the PCR cycles were as follows: 94°C for 2 min 45 s followed by 40 cycles (94°C for 1 min, 60°C for 1 min and 72°C for 1 min 40 s), followed by a final elongation at 72°C (10 min). A touchdown (−0.5°C for annealing temperature over the first 20 cycles) was specifically added for the SCARs derived from RAPDs generated with OPO05 and the OPC20, followed by 20 cycles at 60°C. The newly developed SCAR primers were designated with the *NZsc_*prefix, except for the second set of OPO05 primers, which were designated with *NZsn_* prefix because they are a single nucleotide polymorphism (SNP) marker.

Genetic marker identification and map construction around WAA resistance genes

One hundred and twenty four seedlings of family 1A , 178 seedlings of family 2F and 130 seedlings of family 3A were used for marker identification and mapping of the *Er1*, *Er2* and *Er3* genes, respectively (Table 4). The mapping strategy used was that of the double pseudo-testcross (Grattapaglia and Sederoff 1994). Linkage analysis and genetic map construction for the *Er1* and *Er3* genes were performed using the 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. The localized genetic maps were aligned to the 'Discovery'×TN10-8 (D×T) reference framework map (Calenge et al. 2004) to indirectly determine the positions of both genes on the apple genome. The D×T map has transferable microsatellite markers as its backbone and was developed to map quantitative trait loci for resistance to several important apple diseases (Calenge et al. 2004, 2005a) as well as markers derived from resistance gene analogues (Calenge et al. 2005b). The molecular markers that had been located around the *Er1* and *Er3* genes (mostly SCARs) were employed to genotype the entire D×T population of 149 plants. The microsatellite markers that mapped on the same linkage group (LG) as these markers were reciprocally screened over the original mapping population segregating for *Er3*, plus the validation population segregating for *Er1* (see below). PCR amplifications for the microsatellites were performed as described in the original publications (Calenge et al. 2005b; Silfverberg-Dilworth et al. 2006), and PCR products were resolved using a capillary electrophoresis system (CePRO 9600, Combisep, Ames, IO). In addition, a new set of microsatellite markers was developed from apple expressed sequence tags (Newcomb et al. 2006). For the existing microsatellites (Liebhard et al. 2002; Silfverberg-Dilworth et al. 2006), the nomenclature used by the original authors was retained. For the new microsatellite markers, the prefix *NZms_* was coupled with the GenBank accession number. The *Er2* gene was directly mapped using a saturated genetic map of the M.9×'Robusta 5' progeny (family 2F) developed for the mapping of the dwarfing gene *DW1*

Table 3 The sequences and annealing temperatures of the polymerase chain reaction primers used for the sequence characterised amplified region (SCAR), single nucleotide polymorphism (SNP) and simple sequence repeat (SSR) markers linked to woolly apple aphid (WAA) resistance genes

Marker name	Marker type	Original RAPD/EST	WAA gene	Forward primer	Reverse primer	Product size (bp)	PCR conditions[a]	Linkage group
NZsc_C20	SCAR	OPC20	*Er1*	TCTCTAACTCAATAACTCCCAAGAC	ACTTCGCCACCATTATCACTCCTGA	2,000	Td 70–60	8
NZsc_GS327	SCAR	GS327	*Er1*	GCCAAGCTTCAATGTCGGAGTAGAT	CAAGCTTCCCCTAAGGCTATTGCCA	1,600	Ta 60	8
NZsc_O05	SCAR	OPO05	*Er1*	CCCAGTCACTAACATAATTGGCACA	CCCAGTCACTGGCAAGAGAAATTAC	1,700	Ta 60	8
NZsn_O05	SNP	OPO05	*Er1*/*Er3*	AACGTCATGTCAATAT	CCCAGTCACTGGCAAGAGAAATTAC	880	Td 70–60	8
NZsc_E01	SCAR	OPE01	*Er3*	CCCAAGGTCCGAACACAAATGAGAG	CCCAAGGTCCAAAACTATCCCGAAG	1,350	Ta 60	8
NZsc_A01	SCAR	OPA01	*Er3*	CAGGCCCTTCAGCAAAGAGGTGTCT	CAGGCCCTTCACTACTAATAAGAAC	1,250	Ta 60	8
NZms_EB106753	SSR	EB106753	*Er1*/*Er3*	TCTGAGGCTCCCAAGTCC	TAGGAGCAGAAGAGGTGACG	175	Td 65–60	8
NZms_EB145764	SSR	EB145764	*Er2*	TTCCAGCGATCCAAAACAAT	GCTCAGGAACACCTCGTTCT	198	Td 65–60	17

The SCAR and SNP primers were derived from RAPD markers (Operon Technologies, Alameda, CA), and the SSR primers were designed from ESTs of apple.
[a] *Td* Touchdown PCR, *Ta* annealing temperature.

(Celton et al. 2006). This map was constructed using a total of 134 markers including 90 published microsatellite markers, which enabled alignment with previously published maps (Liebhard et al. 2002; Silfverberg-Dilworth et al. 2006).

Genetic marker validation and MAS

To validate the markers in double-blind tests for their suitability for MAS, markers NZsc_GS327 and NZsc_O05 were screened over 112 randomly selected seedlings of family 1C segregating for *Er1*. Eighty seedlings of family 2A segregating for *Er2* were evaluated with marker NZms_EB145764. One hundred and twenty seedlings of family 3H segregating for *Er3* were screened with marker NZsn_O05. The DNA marker analyses and phenotyping were performed by different personnel on a 'double-blind' basis.

For the MAS, a population segregating for both the *Er1* and *Er3* genes was developed from a cross between 'Northern Spy' and S26R01T053. The progeny of 464 individuals was planted in 1999 and phenotyped in the glasshouse in 2000, applying the same six-point scale used for the single gene families (Table 2A). DNA extracted from 374 seedlings was screened with the NZsn_O05 marker.

Results

Genetics of WAA resistance

The families of all three genes studied showed strong bimodal phenotypic segregation patterns as expected for major genes (Table 4). They were most evident for the *Er3* gene; that is, the majority of the progeny in each family were assigned to either class 0 for immunity or class 5 for high susceptibility (Table 4). However, the *Er1* and *Er2* families showed weaker bimodal segregation patterns, as a relatively large proportion of the seedlings in each family was assigned to class 1 because of the presence of some WAA and/or galls. Only families 1B and 1E of the six families segregating for the *Er1* gene and family 2D of the six families segregating for the *Er2* gene showed phenotypic segregation ratios that did not differ significantly from the resistant to susceptible (R:S) ratio of 1:1 (Table 4). Of the families segregating for the *Er3* gene, about half did not differ significantly from the R:S=1:1 ratio expected for major resistance genes. The test for heterogeneity comprising all families for each of the genes did not support the hypothesis that each of the three WAA resistance genes were major genes (data not presented) because of some families showing severe segregation distortions. Therefore,

Table 4 Phenotypic data of the families segregating for three woolly apple aphid resistance genes: *Er1* from 'Northern Spy,' *Er2* from 'Robusta 5' and *Er3* from 'Aotea 1'

Family	WAA score						Total	Segregation		χ^2	*P**
	0	1	2	3	4	5		R[a]	S[a]	R:S=1:1	
Er1											
1A	49	6	13	18	28	21	135	49	86	10.14	0.00
1B	91	20	8	3	2	61	185	91	94	0.05	0.83
1C	164	17	15	16	5	188	405	164	241	14.64	0.00
1D	27	34	14	18	4	72	169	27	142	78.25	0.00
1E	183	19	9	15	55	117	398	183	215	2.57	0.11
1F	95	7	6	10	15	30	163	95	68	4.47	0.03
	pooled χ^2 (excluding 3 families with $\chi^2>10$)						746	369	377	0.09	0.77
	heterogeneity (*df*=2)									7.01	0.03
Er2											
2A	62	8	6	4	9	8	97	62	35	7.52	0.01
2B	84	27	2	8	6	13	140	84	56	5.60	0.02
2C	80	22	0	4	9	75	190	80	110	4.74	0.03
2D	72	35	7	6	4	29	153	72	81	0.53	0.47
2E	54	43	6	5	2	30	140	54	86	7.31	0.01
2F	133	4	8	5	6	15	171	133	38	52.78	0.00
	pooled χ^2 (excluding 1 family with $\chi^2>10$)						720	352	368	0.36	0.55
	heterogeneity (*df*=4)									19.74	0.00
Er3											
3A	185	0	0	0	5	172	362	185	177	0.18	0.67
3B	20	1	1	0	1	25	48	20	28	1.33	0.25
3C	35	1	1	2	2	14	55	35	20	4.09	0.04
3D	96	3	5	2	1	38	145	96	49	15.23	0.00
3E	40	2	2	6	3	24	77	40	37	0.12	0.73
3F	23	12	3	1	2	8	49	23	26	0.18	0.67
3G	18	9	0	1	2	22	52	18	34	4.92	0.03
3H	51	2	1	6	24	214	298	51	247	128.91	0.00
3I	52	0	0	3	12	157	224	52	172	64.29	0.00
3J	49	7	5	19	24	139	243	49	194	86.52	0.00
3K	77	0	4	10	9	153	253	77	176	38.74	0.00
3L	33	1	5	5	4	15	63	33	30	0.14	0.71
3M	76	0	7	16	7	28	134	76	58	2.42	0.12
3N	29	7	3	10	9	7	65	29	36	0.75	0.39
3O	36	6	4	3	2	18	69	36	33	0.13	0.72
	pooled χ^2 (excluding 5 families with $\chi^2>10$)						897	455	423	2.90	0.09
	heterogeneity (*df*=9)									11.25	0.26

[a] *R* Immunity (class 0), *S* susceptibility (classes 1–5).
*$P(\chi^2>3.84)=0.05$.

the test for heterogeneity was calculated excluding those families that showed high χ^2 values, i.e. higher than 10 (Table 4). While the pooled data for each of the genes did not differ significantly from the expected R:S=1:1 ratio, both the *Er1* and *Er2* families still showed equal numbers of families with significant segregation distortions towards either resistance or susceptibility. Five out of the eight *Er1* and *Er3* progenies with the extreme segregation distortions (families 1A, 1C, 1D and 3H to 3K) were skewed towards susceptibility. However, the two progenies (families 3N and 3O) derived from resistant selections from the strongly skewed family 3H showed segregations that did not differ significantly from R:S=1:1 (Table 4). The same applied to family 1E, while family 1F showed a much smaller segregation distortion than family 1A from which the A108 accessions were selected and was strongly skewed towards susceptibility (Table 4).

The M.9×'Aotea 1' (family 3A) cross was repeated because this family had been screened for resistance to crown rot (*Phytophthora cactorum*) at an early stage before the WAA screening. However, the R:S segregation ratios of families 3A and 3B did not differ from the expected 1:1 ratio, which confirms that the screening for *Phytophthora* resistance had not affected the segregation of WAA

resistance in family 1A, nor was there any evidence in the reciprocal crosses (families 3A to 3G) for parental effects on the segregation distortions for the *Er3* gene (Table 4). In contrast, the R:S segregation ratio of the repeated 'Northern Spy'×'Braeburn' cross (family 1D) was more skewed to susceptibility than in family 1B, as relatively more seedlings were phenotyped to classes 1 to 3 (Table 4).

Bulked segregant analysis and conversion of RAPDs

Screening of the DNA bulks from phenotypically extreme progeny from families segregating for *Er1* and *Er3* was successful for the identification of specific PCR products linked to each of the WAA resistance genes. For family 1A, a 2,000- and a 1,600-bp product obtained from OPC20 and GS327 primers, respectively, were inherited from 'Northern Spy.' The NZsc_O05 marker strongly co-segregated with the class 0 immunity conferred by *Er1*, as only one out of six class 1 seedlings in this family showed the marker (data not presented). For family 3A, a 1,950- and a 1,700-bp RAPD product obtained from OPE01 and OPO05, respectively, and inherited from 'Aotea 1' were associated with *Er3*-derived immunity. One 1,250-bp RAPD product obtained from OPA01 and derived from 'Aotea 1' was associated with the WAA-susceptible phenotype. These markers were used to genotype sub-populations of families 1C and 3A for linkage analysis and map construction around the two WAA resistance genes (data not shown).

Table 3 lists the primers developed to convert RAPD markers to their corresponding SCAR markers. This conversion was particularly important for the 1,700-bp RAPD product obtained from the OPO05 primer, which was tightly linked to the *Er3* gene but was extremely faint and difficult to score. For NZsc_O05, the use of specific primers constructed using the terminal sequences of the RAPD product resulted in the amplification of a co-dominant marker. The allele expressed by the *Er3* heterozygote 'Aotea 1' corresponded to the size of the original RAPD marker and segregated identically in the seedling population. However, a second forward primer was developed because the first did not show any segregation in the validation screen for *Er3* in family 3A. This second primer (NZsn_O05) was based on a SNP found between the 1,700-bp sequences from 'Aotea 1' and 'Royal Gala' and, in combination with the original reverse primer, produced a 880-bp product associated with immunity derived from both *Er1* and *Er3*. The SCAR markers constructed from the 1,600- and 2,000-bp products obtained from GS327 and

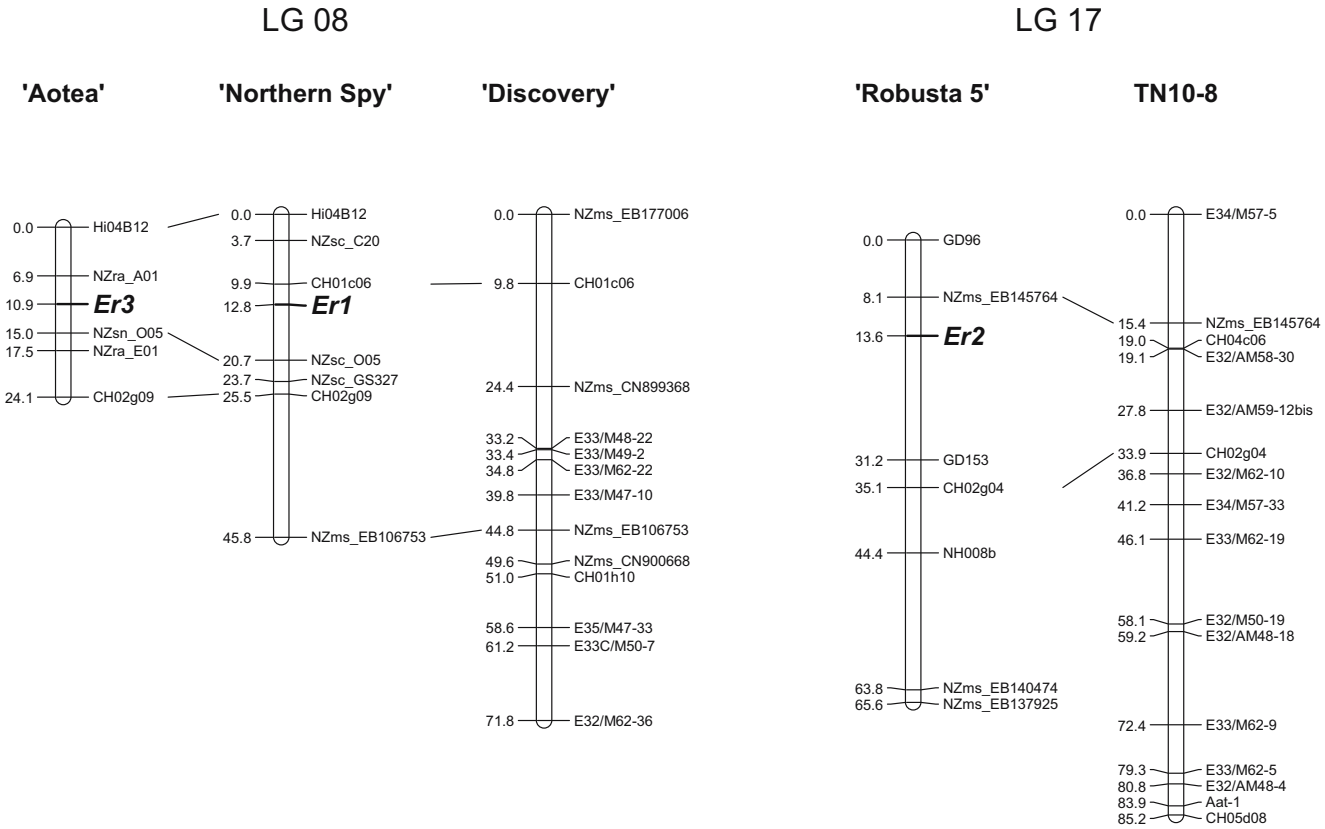

Fig. 1 Genetic maps of the regions around the *Er* genes for woolly apple aphid resistance. The genetic maps of 'Aotea 1,' 'Northern Spy' and 'Discovery' have been aligned using common microsatellite and SCAR markers and correspond to LG 08. The genetic map for *Er1* was constructed using the validation population 'Northern Spy'× 'Royal Gala' (family 1C). The genetic maps of 'Robusta 5' and TN10-8 have been aligned using common microsatellite markers and correspond to LG 17

OPC20 RAPD primers, respectively, had the same size as the RAPD PCR products, were linked to the *Er1* gene and segregated in a dominant/recessive manner. Co-segregation with the resistant phenotype was observed when NZsc_O05 was screened over the *Er1* population. None of the SCAR markers developed segregated in the D×T population; hence, new markers were required to map the genes.

Genetic maps around the three *Er* genes and comparison with a reference map

Two EST-based markers (NZscDR033885 and NZscDR033887) developed from the RFLP screening of candidate resistance genes (Gardiner et al. 2006) and one microsatellite marker (NZms_EB106753) developed in this study were screened over both the *Er1* and *Er3* populations and were found to be linked to the *Er1* gene. These markers were polymorphic in the D×T population and mapped to LG 08. Hence, published microsatellite markers located on LG 08 (Liebhard et al. 2002; Silfverberg-Dilworth et al. 2006) were screened over both the *Er1* and *Er3* populations (i.e. families 1C and 3A, respectively). Two of them (Hi04b12 and CH02g09) mapped in both populations (Fig. 1), which made it possible to compare the genome locations of *Er1* and *Er3* with each other and with the 'Discovery' map. Genetic maps comprising RAPD, SCAR and microsatellite markers were constructed for the resistant parents 'Northern Spy' (*Er1*) and 'Aotea 1' (*Er3*), spanning 24.1 and 45.8 cM, respectively. *Er1* mapped between CH01c06 and NZsc_O05, and *Er3* was flanked by NZsn_O05 and NZra_A01. Alignment of these partial maps with the 'Discovery' map demonstrated that *Er1* and *Er3* mapped to the top half of LG 08. Overall, the marker order and distances were conserved between the three maps.

The *Er2* gene mapped to the top of LG 17 of the saturated genetic map (Fig. 1) constructed from the progeny of family 2F (Celton et al. 2006). Alignment of this map with that of TN10-8 provided further evidence of *Er2* mapping to LG 17.

Validation of *Er* gene markers and MAS

To determine the usefulness of the markers identified in this study for MAS, they were validated in one segregating family each for the three WAA resistance genes. Using family 1C to validate two *Er1* markers, NZsc_O05 and NZsc_GS327 correctly predicted 69 and 70, respectively, of the 77 seedlings that were immune (i.e. class 0) to WAA (Table 5a). However, in contrast to the cross with 'Sciglo' (family 1A) used to identify both markers, all six seedlings with the class 1 phenotype of family 1C showed both markers (Table 5a), hence providing a strong argument that these seedlings do carry the *Er1* gene. Assuming this was

Table 5 Genetic marker validation for three woolly apple aphid resistance genes: A) *Er1* from 'Northern Spy' (family 1C), B) *Er2* from 'Robusta 5' (family 2A) and C) *Er3* from 'Aotea 1' (family 3H)

	Woolly apple aphid phenotype						Segregation[a]		Total
	0	1	2	3	4	5	R	S	
A									
Number of seedlings	77	6	2	0	2	25	83	29	112
NZsc_GS327									
+	69	6	1	0	0	1	75	2	77
−	8	0	1	0	2	24	8	27	35
NZsc_O05									
+	70	6	1	0	1	1	76	3	79
−	7	0	1	0	1	24	7	26	33
B									
Number of seedlings	42	3	6	4	8	17	42	38	80
NZms_EB145764									
+	38	0	0	0	0	1	38	1	39
−	4	3	6	4	8	16	4	37	41
C									
Number of seedlings	20	2	0	0	13	85	20	100	120
NZsn_O05									
+	18	0	0	0	0	1	18	1	19
−	2	2	0	0	13	84	2	99	101

[a] For *Er1*, *R* resistant (classes 0–1), *S* susceptible (classes 2–5); for *Er2* and *Er3*, *R* resistant (class 0), *S* susceptible (classes 1–5).

Table 6 Marker-assisted selection (MAS) with the NZsn_O05 marker for pyramided woolly apple aphid resistance genes *Er1* and *Er3* in a 'Northern Spy' (a_1b)×S26R01T053 (a_3c) family

Genotype	Woolly apple aphid phenotype						Total	Percent correct predictions[a]
	0	1	2	3	4	5		
a_1a_3	34	1	0	0	1	2	38	92.1
a_3b	29	2	0	1	1	9	42	73.8
a_1c	102	25	2	2	3	12	146	87.0
bc	23	6	4	1	5	109	148	80.4
Total	188	34	6	4	10	132	374	

The true MAS success rate measured as the percentage correct predictions will need to be validated through testcrosses as the predictions will be overestimated because of unidentified recombination events between the marker and the resistance genes.
[a] Classes 0–1=resistant; classes 2–5=susceptible.

the case, then 96 individuals out of 112 were correctly predicted in family 1C. Using family 2A to validate the *Er2* marker NZms_EB145764, the phenotype of 75 seedlings out of 80 was correctly predicted (Table 5b). Using family 3H to validate the NZsn_O05 marker for *Er3*, it predicted the individual phenotype for 117 out of 120 individuals, although this population presented a severe segregation distortion (Table 5c). None of the in total five class 1 seedlings in families 2A (*Er2*) and 3H (*Er3*) showed the specific marker for each of the genes.

Out of 464 individual seedlings from the 'Northern Spy'×S26R01T053 population used for MAS of seedlings carrying both *Er1* and *Er3*, 74 died and could not be phenotyped. In the remaining 390 individuals, a R:S=196:194 ratio was observed, which differed significantly from the expected R:S=3:1 ratio (χ^2=127.3; $P \leq 0.001$). In total, 374 individuals were screened with the NZsn_O05 marker that is close to both *Er1* and *Er3* (Fig. 1). Three segregating alleles were obtained, with allelotype *ab* being assigned to 'Northern Spy' and *ac* to S26R01T053. While the *a* alleles from both resistant hosts were indistinguishable with this SNP marker, their parental origin can be deduced because of the three-allele situation. To visualize the gametic recombination in the meiotic phase, subscripts have been added to ascribe the origin of the *a* alleles (a_1 from 'Northern Spy' and a_3 from S26R01T053). With the four allelotypes a_1a_3, a_3b, a_1c and *bc* represented by 38, 42, 146 and 148 individuals, respectively (Table 6), the low number of seedlings carrying the a_3 allele shows that there has been a major segregation distortion associated with the *Er3* gene. The genotypes were compared to the phenotypes to determine the success rate of MAS in identifying individuals that carry both WAA resistance genes. For this purpose, with 25 progeny of the a_1c allelotype being assigned to class 1 while having the a_1 marker allele indicating the presence of the *Er1* gene, all class 1 progeny of the 'Northern Spy'×S26R01T053 family were assumed to carry the gene. As a result, only 38 individuals (10% of the population) showed the a_1a_3 allelotype. However, with 35 (92%) of these progeny being WAA resistant (classes 0 and 1), MAS was very effective in identifying the individuals expected to carry both the *Er1* and *Er3* genes (Table 6). This was a much better prediction rate than for the seedlings having one of the other three allelotypes and particularly those that carry the *b* allele of the NZsn_O05 marker (Table 6).

Discussion

Phenotyping and genetics of WAA resistance

This paper presents the findings of in-depth studies of the genetics of inheritance of three sources of WAA resistance: 'Northern Spy' (*Er1*), 'Robusta 5' (*Er2*) and 'Aotea 1' (*Er3*) over a number of families. The strong resistance in the form of immunity to WAA infection combined with the bimodal segregation pattern of this in the progenies suggested the presence of major genes for resistance in these accessions. However, the segregation ratios of many progenies derived from the three sources of resistance differed significantly from the expected R:S=1:1 ratio, when the immune seedlings were classified as resistant and those with any number of aphids and/or galls were classified as susceptible. These classifications are arbitrary, and the dividing line could have been placed between classes 2 and 3, as was done by Knight et al. (1962) to argue the presence of a major WAA resistance gene in 'Northern Spy.' While this would have indeed improved the segregation ratios for some progenies (e.g. families 1A, 1C, 1D and 2C), it would also have further skewed the segregation ratios of other families already strongly skewed towards resistance (e.g. families 1F, 2A, 2B, 2F and 3C). The presence of progeny with an intermediate level of resistance may be best explained by quantitative resistance factors in the background of particularly the *Er1* and *Er2* genes in 'Northern Spy' and 'Robusta 5,' respectively. At the same time, we have shown that segregation distortions

are common and vary among families for the three *Er* genes studied here. This is in agreement with Knight et al. (1962), who found that their M.9×'Northern Spy' families segregating for *Er1* were very strongly skewed towards susceptibility. Similarly, with only 10% immune and a further 25% resistant seedlings, the families of 'Robusta 5' crossed with the rootstocks M.9, and M.27 segregating for *Er2* were also skewed towards susceptibility (Cummins and Aldwinckle 1983).

Other factors that can affect segregation ratios include biological explanations, such as the presence of biotypes and variable phenotyping conditions. Biotypes that partially overcome major resistance genes have been demonstrated for the *Er1* gene in the USA (Gambrell and Young 1950; Rock and Zeiger 1974; Young et al. 1982), South Africa (Giliomee et al. 1968) and Australia (Sen Gupta and Miles 1975), and for the *Er3* gene in New Zealand (Sandanayaka et al. 2003). In the latter case, the biotype appears to have been present only temporarily, as the biotype has not been found in resistance screenings in recent years. 'Spy-capable' WAA still appear to be present in South African orchards but have not rendered the rootstocks carrying *Er1* completely susceptible (Pringle and Heunis 2001). The existence of biotypes has also been used to explain the variable performance of some apple accessions with regard to WAA resistance (Knight et al. 1962). The temporary ability of WAA to infest resistant rootstocks therefore may be better explained by variations in environmental conditions affecting the expression of the resistance genes. In our experiments, the protocol for WAA screening in both the glasshouse and field involved infestation of plant material in January to February and resistance assessment in March to April. This time frame resembles the normal seasonal development of WAA in New Zealand. However, because of late seed planting, a different time frame was initially applied to family 1C, where the phenotyping was performed in the glasshouse in late winter in August 2002. This resulted in only 6% of the seedlings being free of aphids and galls, while 44% were assigned to classes 2 and 3, and 42% to class 5. The next year, the phenotyping was repeated, and the standard protocol was applied. By that time, 64% of the progeny classed as 5 in the first screen had died, while 28% of the live seedlings were classed as 5 again; that is, few plants changed phenotypic class. In contrast, 71% of the seedlings that were classed as 2 and 3 in the first screen were classed as 0 in the second screen. The infestation pressure in the first screen was very high, as the conditions were conducive to WAA development after a long inoculation period, but the conditions appear to have been sub-optimal for resistance expression. Another explanation for segregation distortions are the presence of (sub-) lethal genes linked to resistance genes. Such genes have been suggested to explain the segregation distortions that are common with the scab resistance genes *Vf* (Gao and Van de Weg 2006) and possibly the *Vh2* and *Vh8* genes (Bus et al. 2002, 2005).

Our research confirms the presence of a major gene in both 'Northern Spy' (Knight et al. 1962) and 'Robusta 5', which have been named *Er1* and *Er2*, respectively. Although the *Er2* gene initially was assigned to a putative WAA resistance gene in what possibly may have been a susceptible accession, 3762 (King et al. 1991), derived from a different *M.×robusta* accession than 'Robusta 5,' we propose that the primary source of *Er2* is 'Robusta 5'. We also have identified a new major gene for resistance to WAA in the *M. sieboldii* accession 'Aotea 1.' In line with the naming of the first two major genes for WAA resistance (King et al. 1991; Knight et al. 1962), we name this dominant allele *Er3*. The presence of major genes is supported by genetic marker alleles being linked to immunity in 'Robusta 5' and 'Aotea 1' and to immunity/high resistance in 'Northern Spy' (Table 5).

Genome mapping of three WAA resistance genes

To follow up our preliminary identification of markers for the *Er1* and *Er3* genes (Bus et al. 2000b), we have now located three WAA resistance genes on the apple genome. The assignment of the *Er1* and *Er3* genes to the top of LG 08, together with the fact that markers linked to SI on LG 17 did not co-segregate with either *Er1* or *Er3* in our populations (data not shown), confirms recent findings (Tobutt et al. 2000) that previous work suggesting *Er1* is linked to the SI locus (Knight et al. 1962) is incorrect. The segregation distortions found in the 'Northern Spy' and 'Aotea 1' progenies therefore have to be attributed to other causes. Similarly, we have found that the *Er2* gene maps to the top of LG 17 at the opposite end to the SI locus, which implies that the strong segregation distortions observed are not due to self-incompatibility. Recently, three nucleotide binding site–leucine-rich repeat genes have been mapped to the same region (Calenge et al. 2005b) as *Er2*, which may provide further markers for this WAA resistance gene.

Although *Er1* and *Er3* map to the same genomic region, the fact that a biotype of WAA was able to overcome *Er1* but not *Er3* (Sandanayaka et al. 2003) confirms that they are different but closely linked loci or alleles of the same locus with different functionality. Further studies involving more closely linked markers may help to answer this question. Moreover, the mapping of a powdery mildew quantitative trait locus co-locating with resistance gene analogues on the 'Discovery' map (Calenge et al. 2005b) and a major gene for powdery mildew resistance (*Pl-w*) (Evans and James 2003) in the same region on LG 08 as *Er1* and *Er3* suggests that there is a significant cluster of resistance genes in this chromosomal region. In addition,

the *Vfh* gene for apple scab resistance and the *Dp-fl* gene for rosy apple aphid resistance (Durel 2006) both mapped more distantly to the *Er* genes on the same LG, which makes this chromosome a good candidate for complete sequencing, with the ultimate goal of cloning those five resistance genes.

Marker-assisted selection and gene pyramiding

A major focus of the New Zealand apple breeding programme is the rapid development of new cultivars with durable resistances to multiple pests and diseases. The validation of the flanking markers in secondary *Er1* and *Er3* populations using a double-blind test shows that they are effective tools for selecting resistant seedlings in the large seedling progenies required for cultivar development. In the 'Northern Spy'-derived family 1C, the phenotype of only 10 (9%) out of 112 seedlings failed to be correctly predicted using the markers flanking *Er1*. This is an excellent prediction rate considering that the closest (NZsc_O05) of the two markers used is linked to this gene at about 8 cM (Fig. 1). However, the resistant seedlings included the class 1 seedlings as they showed the marker, which was in contrast to the cross with 'Sciglo,' family 1A, where only one out of six of the class 1 seedlings showed the marker. This may be attributed to the different screening conditions applied to the two families, with family 1A having been screened in the field and family 1C in the glasshouse, which supports the suggestion that the resistance expression of this gene is influenced by environmental conditions as discussed above. In addition, genes may be expressed at different levels in different genetic backgrounds under the same environmental conditions as was demonstrated with the *Vf* gene for scab resistance (Gardiner et al. 1996). The NZsc_O05 marker was identified using DNA bulks of immune seedlings, but with the marker sometimes also being present in class 1 progeny of *Er1* families, genotypic selection will be more consistent than phenotypic selection that is sensitive to environmental conditions.

As a step towards the goal of developing plants with two or more pyramided resistances to WAA infestation, we screened a population from a 'Northern Spy' (*Er1*)× S26R01T053 (*Er3*) cross using the NZsn_O05 marker. We found 16.6% of recombinants between this marker and the resistance genes when regarding classes 0 and 1 as resistant and classes 2 to 5 susceptible. Because 28 out of 34 seedlings of class 1 (Table 6) carried the *a* allele of NZsn_O05, which predominantly was due to 20% of the seedlings with marker genotype *ac* being class 1 indicating the presence of *Er1*, these seedlings were classed as resistant. With a relatively low percentage of 73.8% of the *ab* and 80.4% of the *bc* genotypes predicted correctly for their WAA resistance phenotype, the higher recombinant rates predominantly involved seedlings carrying the *b* allele of the NZsn_O05 marker that originated from 'Northern Spy' (Table 6). Perhaps this may be attributed to the phenotyping having been less effective in 1999 when this family was screened. Research with genetic markers on family 1E, which also segregates for *Er1* and was screened in the same year, confirmed this.

The tri-allelic nature of the NZsn_O05 marker (i.e. *ab*× *ac*) made it possible to identify 35 WAA-resistant individuals that are expected to carry both the *Er1* and *Er3* genes based on their *aa* genotype, making them potentially highly suitable breeding parents. As no colonies of the *E. lanigerum* biotype overcoming the *Er3* (Sandanayaka et al. 2003) or the *Er1* genes were available to phenotype the progeny, back-crossing of the selected progeny will be required to ascertain they all carry both resistance genes. However, 38 out of 222 resistant progeny (16%) with the *aa* genotype for NZsn_O05 was much lower than expected because of segregation distortion associated with the *Er3* gene. The progeny was strongly skewed towards susceptibility as a result of many *aa* and *ab* genotypes having been lost, which, as discussed above, may be explained by the *Er3* locus being linked to (sub-)lethal genes. Segregation distortions were present but were not so strong in the *Er1* and *Er2* accessions.

To maintain gene combinations involving linked resistance genes such as *Er1* and *Er3* as described for the 'Northern Spy' (*Er1*)×S26R01T053 (*Er3*) cross in breeding parents, a breeding strategy needs to be put in place because the selections will carry the genes in repulsion phase and therefore will dissociate in further crossings. One way of facilitating the back-cross process is to select for recombinants carrying both genes in coupling phase (Bus et al. 2004).

In summary, the present study has identified a new WAA resistance gene, *Er3*, in addition to the previously known *Er1* and *Er2* genes, and molecular markers suitable for MAS in breeding populations have been developed for all three resistances. The distances between the closest markers identified in the present study and the WAA resistance genes do not allow us to envisage the cloning of those genes at this time. Denser genetic maps around these genes need to be developed, and this should be made possible by the development of more molecular markers. At least 250,000 apple ESTs exist in public databases (Newcomb et al. 2006) and many potential markers, such as microsatellites or SNPs, will help improve the effectiveness of MAS (Gardiner et al. 2007).

Other sources of WAA resistance are being studied to widen the genetic base and to increase the probability of breeding durable resistance to this pest. For example, partial WAA resistance of several large-fruited apple cultivars has

been investigated (Sandanayaka et al. 2005), and new sources of WAA resistance have been identified in a breeding population (Alspach and Bus 1999) set up to increase the genetic diversity of apple in New Zealand (Noiton and Shelbourne 1992). Genetic marker studies have been initiated on several accessions selected from this germplasm that carry major genes for WAA resistance (Bus et al. 2000a).

Acknowledgements This research was supported in part by grants from ENZAFRUIT New Zealand (International), PREVAR™ and the New Zealand Foundation for Research, Science and Technology. The authors thank Mike Cook and Wendy Legg for their help with leaf collection, DNA preparation and marker analysis.

References

Alspach PA, Bus VGM (1999) Spatial variation of woolly apple aphid (*Eriosoma lanigerum*, Hausmann) in a genetically diverse apple planting. NZ J Ecol 23:39–44

Alston FH (1977) Practical aspects of breeding for mildew (*Podosphaera leucotricha*) resistance in apples. In: Proc. Eucarpia Fruit Section Symposium VII, Top Fruit Breeding, Wageningen, 1976, pp 4–13

Alston FH (1983) Progress in transferring mildew (*Podosphaera leucotricha*) resistance from *Malus* species to cultivated apple. IOBC/WPRS Bull 6(4):87–95

Alston FH, Briggs JB, Bates JW (1971) Pest resistance. East Malling Report for 1970:95

Alston FH, Phillips KL, Evans KM (2000) A *Malus* gene list. Acta Hortic 538:561–570

Brown MW, Schmitt JJ (1990) Growth reduction in nonbearing apple trees by woolly apple aphids (Homoptera: Aphididae) population. J Econ Entomol 83:1526–1530

Brown MW, Schmitt JJ, Ranger S, Hogmire HW (1995) Yield reduction in apple trees by edaphic woolly apple aphids (Homoptera: Aphididae) populations. J Econ Entomol 88:126–133

Bus V, Bradley S, Hofstee M, Alspach P, Brewer L, Luby J (2000a) Increasing genetic diversity in apple breeding to improve the durability of pest and disease resistance. Acta Hortic 538:185–190

Bus V, Ranatunga C, Gardiner S, Bassett H, Rikkerink E (2000b) Marker assisted selection for pest and disease resistance in the New Zealand apple breeding programme. Acta Hortic 538:541–547

Bus V, White A, Gardiner S, Weskett R, Ranatunga C, Samy A, Cook M, Rikkerink E (2002) An update on apple scab resistance breeding in New Zealand. Acta Hortic 595:43–47

Bus V, van de Weg WE, Durel CE, Gessler C, Calenge F, Parisi L, Rikkerink E, Gardiner S, Patocchi A, Meulenbroek B, Schouten H, Laurens F (2004) Delineation of a scab resistance gene cluster on linkage group 2 of apple. Acta Hortic 663:57–62

Bus VGM, Laurens FND, Van de Weg WE, Rusholme RL, Rikkerink EHA, Gardiner SE, Bassett HCM, Kodde LP, Plummer KM (2005) The *Vh8* locus of a new gene-for-gene interaction between *Venturia inaequalis* and the wild apple *Malus sieversii* is closely linked to the *Vh2* locus in *M. pumila* R12740-7A. New Phytol 166:1035–1049

Calenge F, Faure A, Goerre M, Gebhardt C, Van De Weg WE, Parisi L, Durel C-E (2004) Quantitative Trait Loci (QTL) analysis reveals both broad-spectrum and isolate-specific QTL for scab resistance in an apple progeny challenged with eight isolates of *Venturia inaequalis*. Phytopathology 94:370–379

Calenge F, Drouet D, Denancé C, Van De Weg WE, Brisset M-N, Paulin J-P, Durel C-E (2005a) Identification of a major QTL together with several minor additive or epistatic QTLs for resistance to fire blight in apple in two related progenies. Theor Appl Genet 111:128–135

Calenge F, Van Der Linden CG, Van De Weg E, Schouten HJ, Van Arkel G, Denancé C, Durel C-E (2005b) Resistance gene analogues identified through the NBS-profiling method map close to major genes and QTL for disease resistance in apple. Theor Appl Genet 110:660–668

Celton J-M, Rusholme R, Tustin S, Ward S, Ambrose B, Ferguson I, Gardiner S (2006) Genetic mapping of DW1, a locus required for dwarfing of apple scions by 'M.9' rootstock. In: Abstract book of the 3rd International Rosaceae Genomics Conference, 19–22 March 2006, Napier, New Zealand, p 39

Crane MB, Greenslade RM, Massee AM, Tydeman HM (1936) Studies on the resistance and immunity of apples to the woolly apple aphid, *Eriosoma lanigerum* (Hausm.). J Pomol Hortic Sci 14:137–163

Cummins JN, Aldwinckle HS (1983) Breeding apple rootstocks. Plant Breed Rev 1:294–394

Cummins JN, Forsline PL, Mackenzie JD (1981) Woolly apple aphid colonization on Malus cultivars. J Am Soc Hortic Sci 106:26–30

Dozier WA, Latham AJ, Kouskolekas CA, Mayton EL (1971) Susceptibility of certain apple rootstocks to black root rot and woolly apple aphids. HortScience 9:35–36

Dunemann F, Urbanietz A, Gardiner S, Bassett H, Legg W, Rusholme R, Bus V, Ranatunga C (2004) Marker assisted selection for Pl-1 powdery mildew resistance in apple—old markers for a new resistance gene? Acta Hortic 663:757–762

Durel C-E (2006) Genetic localisation of new major and minor pest and disease factors in the apple genome. Rosaceae Genomics Conference 3, Napier, New Zealand

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

Gambrell FL, Young HC (1950) Habits, rates of infestation and control of woolly apple aphid in nursery plantings. J Econ Entomol 43:463–465

Gao ZS, Van de Weg WE (2006) The Vf gene for scab resistance in apple is linked to sub-lethal genes. Euphytica 151:123–132

Gardiner SE, Bassett HCM, Noiton DAM, Bus VG, Hofstee ME, White AG, Ball RD, Forster RLS, Rikkerink EHA (1996) A detailed linkage map around an apple scab resistance gene demonstrates that two disease resistance classes both carry the Vf gene. Theor Appl Genet 93:485–493

Gardiner SE, Bus VG, Chagné D, Ranatunga C, Legg W, Bassett HCM, Zhou J, Cook M, Crowhurst RN, Gleave AP, Rikkerink EHA, Patocchi A, Durel C-E (2006) Mapping of major resistances to woolly apple aphid. In: Plant and Animal Genome Conference XIV, San Diego, CA, USA, 14-18 January 2006. http://www.intl-pag.org/14/abstracts/PAG14_P495.html

Gardiner SE, Bus VGM, Rusholme RL, Chagné D, Rikkerink EHA (2007) Apple. In: Kole C (ed) Genome mapping and molecular breeding, vol. 4. Springer, Heidelberg, pp 1–62

Giliomee JH, Strydom DK, Van Zyl HJ (1968) Northern Spy, Merton and Malling–Merton rootstocks susceptible to woolly aphid, *Eriosoma lanigerum*, in the Western Cape. South Afr J Agric Sci 11:183–186

Grattapaglia D, Sederoff R (1994) Genetic linkage maps of *Eucalyptus grandis* and *Eucalyptus urophylla* using a pseudo-testcross: mapping strategy and RAPD markers. Genetics 137:1121–1137

Jancke O (1937) Ueber die Blutlausanfälligkeit von Apfelsorten, wilden Malusarten und—bastarden, sowie die Züchtung blutlausfester Edeläpfel und Unterlagen. Phytopathol Z 10:184–196

King GJ, Alston FH, Battle I, Chevreau E, Gessler C, Janse J, Lindhout P, Manganaris AG, Sansavini S, Schmidt H, Tobutt KR (1991) The 'European Apple Genome Mapping Project'—developing a strategy for mapping genes coding for agronomic characters in tree species. Euphytica 56:89–94

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

Knight RL, Briggs JB, Massee AM, Tydeman HN (1962) The inheritance of resistance to woolly aphid, *Eriosoma lanigerum* (Hausm.), in the apple. J Hortic Sci 37:207–218

Lemoine J, Huberdeau D (1999) Le puceron lanigerère (*Eriosoma lanigerum* Hausmann), un parasite en recrudescence dans les vergers de pommiers. Arboric Fruit 532:19–26

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

Mackenzie JD, Cummins JN (1982) Differentiation of *Malus* clones into resistance classes by their effects on the biology of *Eriosoma lanigerum* Hausmn. J Am Soc Hortic Sci 107:737–740

Manganaris AG, Alston FH (1987) Inheritance and linkage relationships of glutamate oxaloacetate transaminase isoenzymes in apple 1. The gene GOT-1, a marker for the S incompatibility locus. Theor Appl Genet 74:154–161

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

Newcomb RD, Crowhurst RN, Gleave AP, Rikkerink EHA, Allan AC, Beuning LL, Bowen JH, Gera E, Jamieson KR, Janssen BJ, Laing WA, McArtney S, Nain B, Ross GS, Snowden KC, Souleyre EJ, Walton EF, Yauk YK (2006) Analyses of expressed sequence tags from apple. Plant Physiol 141:147–166

Noiton D, Shelbourne CGA (1992) Quantitative genetics in an apple breeding strategy. Euphytica 60:213–219

Paran I, Michelmore RW (1993) Development of reliable PCR-based markers linked to downy mildew resistance genes in lettuce. Theor Appl Genet 85:985–993

Pringle KL, Heunis JM (2001) Woolly apple aphid in South Africa: biology, seasonal cycles, damage and control. Decid Fruit Grow 51(4):22,23,36

Rock G, Zeiger D (1974) Woolly apple aphid infests Malling and Malling–Merton rootstocks in propagation beds in North Carolina. J Econ Entomol 67:137–138

Sandanayaka WRM, Bus VGM, Connolly P, Newcomb R (2003) Characteristics associated with woolly apple aphid Eriosoma lanigerum, resistance of three apple rootstocks. Entomol Exp Appl 109:63–72

Sandanayaka WRM, Bus VGM, Connolly P (2005) Mechanisms of woolly aphid [*Eriosoma lanigerum* (Hausmann)] resistance in apple. J Appl Entomol 129:534–541

Sen Gupta GC, Miles PW (1975) Studies on the susceptibility of varieties of apple to the feeding of two strains of woolly aphis (Homoptera) in relation to the chemical content of the tissues of the host. Aust J Agric Res 26:157–168

Silfverberg-Dilworth E, Matasci CL, Van de Weg WE, Van Kaauwen MPW, Walser M, Kodde LP, Soglio V, Gianfranceschi L, Durel C-E, Costa F, Yamamoto T, Koller B, Gessler C, Patocchi A (2006) Microsatellite markers spanning the apple (*Malus*×*domestica* Borkh) genome. Tree Genetics Genomes 2:202–224

Speyer W (1924) Über die Blutlausanfälligkeit von Apfelsorten. Angew Bot 6:168–171

Taylor JB (1981) The selection of Aotea apple rootstocks for resistance to woolly aphis and to root canker, a decline and replant disease caused by basidiomycete fungi. NZ J Agric Res 24:373–377

Tobutt KR, Boskovic R, Roche P (2000) Incompatibility and resistance to woolly apple aphid in apple. Plant Breeding 119:65–69

Wilton J (1998) Woolly apple aphid: don't turn the clock back 100 years. Orchardist 71(6):11–13

Young E, Rock GC, Zeiger DC, Cummins JN (1982) Infestation of some Malus cultivars by the North Carolina woolly apple aphid biotype. HortScience 17:787–788