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The *Vh2* and *Vh4* scab resistance genes in two differential hosts derived from Russian apple R12740-7A map to the same linkage group of apple

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Abstract

Russian apple R12740-7A is the designation for an accession grown from seed collected in Russia, which was found to be highly resistant to apple scab. The resistance has historically been attributed to a naturally pyramided complex involving three major genes: one race-nonspecific gene, *Vr*, conditioning resistance to all known races, plus two race-specific genes. The race-nonspecific gene was identified as an independently segregating gene by Dayton and Williams (1968) and is referred to in this paper as *Vr*-DW. The first researchers to study the scab resistance gene complex in Russian apple never described the phenotype conditioned by the race-nonspecific gene. Later, Aldwinckle et al. (1976) associated the name *Vr* with a scab resistance gene conditioning distinctive stellate necrotic reactions, which we refer to as *Vr*-A in order to distinguish it from *Vr*-DW. We show that the segregation ratios in progenies from the scab differential hosts 2 and 4 that are derived from Russian apple, crossed with susceptible cultivars were consistent with a single gene conditioning resistance in each host. The genes have been named *Vh2* and *Vh4*, respectively. Resistant segregants from host 2 showed stellate necrotic reactions, while those from host 4 showed hypersensitive reactions. Both the phenotypes and the genetic maps for the genes in the respective hosts were very similar to those of the genes previously named *Vr*-A and *Vx*, respectively, in an F1 family of Russian apple. We showed that race 2 of *V. inaequalis* isolated from host 2 was able to infect resistant descendants of the non-differential accession PRI 442-23 as well as host 2. The descendants of PRI 442-23 were expected to carry the race-nonspecific *Vr*-DW gene, but in fact carry *Vr*-A. We conclude that the *Vh2* gene in host 2 and *Vr*-A are the same, and that the *Vh4* gene in host 4 and *Vx* are the same. However, a major finding of this study is that the latter gene mapped to linkage group 2 of apple instead of linkage group 10 as suggested from previous research. With the two race-specific genes from Russian apple defined

now, we discuss the nature of the race-nonspecific *Vr*-DW gene in this accession. We also report the identification of a new scab resistance gene, *VT57*, from either 'Golden Delicious' or 'Red Dougherty', which conditions chlorotic resistance reactions and is linked to *Vh2*.

Introduction

Apple scab, incited by *Venturia inaequalis* (Cke) Wint., is a major disease requiring extensive control measures in most apple production regions around the world. Resistance breeding is likely to be the major contributor to worldwide efforts to reduce the use of fungicide-based control of the disease. Russian apple R12740-7A (*Malus* Mill. sp.) is a highly scab-resistant accession, which has been the progenitor of many breeding lines in most apple breeding programmes around the world. The name is the designation for a selection grown in the USA from seed obtained from Leningrad, Russia in 1937, and may have had its origins in the Northern Caucasus (Dayton et al. 1953). The scab resistance of Russian apple R12740-7A was initially attributed to a naturally occurring complex involving at least two genes (Dayton et al. 1953), of which the gene that was effective against all known scab races, was designated as the "non-differential", i.e. race-nonspecific, *Vr* gene (Dayton and Williams, 1968; Hough et al. 1970). Later, the complex was extended to include three single genes: the race-nonspecific *Vr* gene, plus two race-specific genes overcome by different isolates of the apple scab fungus (Shay et al. 1962; Williams and Kuć 1969). This generally accepted view was re-worded by Crosby et al. (1992) as follows: "Race 2 … attacks … certain segregates (sic) of the Russian seedling R12740-7A (but not *Vr*)" and "Race 4 … successfully attacks certain segregates of Russian seedling R12740-7A, suggesting a third locus for qualitative resistance different from that overcome by race 2 or *Vr*". Hence the scab resistance genes in the two differential hosts derived from Russian apple were tentatively named *Vh2* and *Vh4*, respectively (Bus et al. 2000), to distinguish them from *Vr* (Dayton and Williams 1968), referred to as *Vr*-DW in this paper, and a further nine genes named at that stage: *Va*, *Vb*, *Vbj*, *Vc*, *Vf*, *Vfh*, *Vg*, *Vj*, and *Vm* (Hough et al. 1953, 1970; Dayton and Williams 1968, 1970; Korban and Chen 1992; Bénaouf and Parisi 2000). The *Vf* gene has been used most extensively in apple breeding programmes around the world (Crosby et al. 1992), but has been overcome by races of *V. inaequalis* (Parisi et al. 1993; Parisi and Lespinasse 1996; Bénaouf and Parisi 2000). These findings accentuate the need for a breeding strategy for durable resistances based on using a diverse range of resistance genes against diseases with a high evolutionary risk of developing virulent races, such as *Venturia inaequalis* (McDonald and Linde 2002).

The high evolutionary risk of *V. inaequalis* developing new races is exemplified by the fact that races able to overcome the resistance genes in certain segregants derived from Russian apple, were already discovered in the early stages of the Purdue-Rutgers-Illinois (PRI) modified back-cross programme in the USA. While race 2 (Shay and Williams 1956) and scab differential host 2 (Williams and Shay 1957) have been described in detail, this is not the case for scab differential host 4 and race 4. The symptoms conditioned by the non-differential host were not described either by the PRI researchers, but about 20 years later the name *Vr* became associated with a gene from Russian apple conditioning stellate necrosis (SN) (Aldwinckle et al. 1976), referred to as *Vr*-A in this paper, although the same symptoms had been shown to be conditioned in host 2 (Williams and Shay 1957). The term SN was coined by Shay and Hough (1952) to describe the distinctive star-shaped reactions to distinguish it from other Class 2 resistance reactions.

Initially, no individual accessions derived from Russian apple were officially proposed to represent the differential hosts once the races had been identified. Hosts 2 and 4 were simply referred to in the literature as the s_i (for race 2) and s_i' (for race 4) segregants from Russian apple (Olivier and Lespinasse 1982; Lespinasse 1989), until they were identified as TSR34T15 and TSR33T239, respectively (Lespinasse, pers. comm.). However, host 2 has erroneously been reported as being accession TSR34T132 from the PRI breeding programme in

a number of publications (Parisi et al. 1993; Parisi and Lespinasse 1996; MacHardy 1996; Bus et al. 2000).

While sequenced characterised amplified region (SCAR) markers have been identified for the scab resistance genes in hosts 2 and 4 (Bus et al. 2000), they have not been assigned to a specific linkage group on the apple map to date. Genetic marker studies on the scab resistance of Russian apple crossed with 'Rome Beauty' revealed that the gene conditioning SN maps to the distal end of linkage group 2 (LG2) (Hemmat et al. 2002), and the gene conditioning a hypersensitive response (HR) (Hemmat et al. 2003b) to LG10 of the European apple map (Maliepaard et al. 1998). Recently, the *Vr1* (Boudichevskaia et al. 2004) and *Vr2* (Patocchi et al. 2004) genes from different Russian apple accessions were mapped to the proximal end of LG2. The many genes named by the different research groups and the fact that both *Vr*-A and *Vh2* have been associated with SN resistance, have created a conundrum about the identities of the scab resistance genes in Russian apple R12740-7A.

In this paper, we characterise hosts 2 and 4 derived from Russian apple R12740-7A in the PRI breeding programme. We present the results of investigations initiated in order to provide answers to questions related to the identity of the scab resistance genes carried by these hosts as well as an accession expected to carry *Vr*-DW from a non-differential segregant of Russian apple. We show that *Vr*-A and *Vh2* are the same gene, and present genetic maps for the regions around the *Vh2* and *Vh4* genes and position them on the apple genome. We also discuss the nature of the third resistance gene in Russian apple based on evidence in the literature, and its relationship to the original race-nonspecific *Vr*-DW gene.

Materials and methods

Studies were performed at HortResearch in New Zealand (NZ) and at Plant Research International in the Netherlands (NL). The reason for performing some of the studies in NL was because they involved races of *V. inaequalis* that are not present in NZ.

Plant material

The NZ studies were performed on seedling progenies derived from two scab differential hosts that had been derived as F2 of Russian apple R12740-7A: TSR34T15 (host 2) and TSR33T239 (host 4). Budwood of these accessions was received from Y. Lespinasse (INRA, Angers, France) under the local designations of X2250 for host 2 and X2249 for host 4. The third parent, A68R03T057 from the HortResearch breeding programme, is derived from the non-differential descendant OR42T175 (PRI 442-23) (Williams and Brown 1968) of Russian apple. Since both the grandparent S80ER28T105 (PRI 1293-100) and parent OB1R2T41 (PRI 2375-5) of A68R03T057 are noted as non-differential hosts (A. Whipkey and J. Janick, pers. comm.), it was expected to carry the *Vr*-DW gene (Figure 1). However, as will become apparent later, the gene in A68R03T057 was actually *Vr*-A and for the sake of clarity in this paper, we will refer to the gene in this host as *Vr*-A, and to the gene in host 2 as *Vh2*. Similarly, we refer to the gene in host 4 as *Vh4*, and the HR conditioning gene mapped by Hemmat et al. (2002, 2003a, b) as *Vx*. The complete pedigrees of the three accessions (Figure 1) were extracted from the website of the PRI breeding programme (http://www.hort.purdue.edu/newcrop/pri/default. html) after receiving the PRI progeny numbers of the accessions from J. Janick and A. Whipkey.

In the period 1999–2001, five F1 progenies were developed with host 2 by crossing it with the scab susceptible accessions 'Royal Gala', Pacific Queen^{TM} (='Gala' × 'Splendour'), A20R01T289, A20R02T032 and A22R11T137 (selections from crosses between 'Braeburn' and 'Royal Gala'). A further two F2 progenies were developed from this host by crossing the resistant accessions AA848 and AA962 from an A163-42 × TSR34T15 family with the scab susceptible host Pacific Beauty^{TM} (='Gala' × 'Splendour'). The A163-42 × TSR34T15 family had been developed to pyramid the *Vf* and the *Vh2* genes. However, testcross progenies of selections AA848 and AA962 from this family with the scab susceptible cultivar Pacific Beauty^{TM} showed SN resistance symptoms only and it was concluded that the two resistant parents only carried the *Vh2* gene. In the period 1998–2001, six F1 families were developed with host 4 crossed with the scab susceptible cultivars

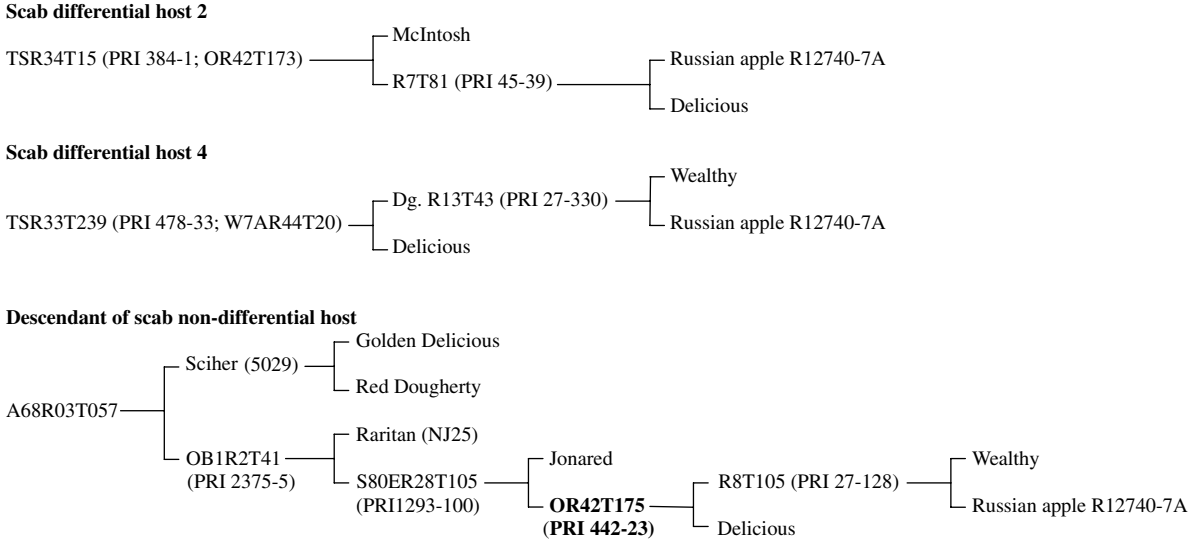

Figure 1. The pedigrees of the scab differential hosts 2 and 4, and accession A68R03T057 derived from Russian apple R12740-7A. A68R03T057 is a descendant from the non-differential host OR42T175 (PRI 442-23) (Williams and Brown 1968). The pedigree information was collated from the website of the Purdue-Rutgers-Illinois apple breeding programme (http://www.hort.purdue.edu/newcrop/pri/default.html).

'Granny Smith', 'Royal Gala', Pacific Queen™, and selections A19R03T127, A22R11T137, and A22R16T173 from crosses between 'Braeburn' and 'Royal Gala'. The seeds from the crosses were planted in root trainer baskets with seed-raising mix containing a general slow-release fertiliser. The seed-raising mix was saturated with water and the baskets were placed in a bin lined with plastic. The seed were stratified for 6–8 weeks at 0–1 °C in a coolstore, followed by forcing in the greenhouse.

For the host-pathogen interaction study in NL, progenies of A68R03T057 crossed with Southern Snap™ (='Gala' × 'Splendour'), and TSR34T15 crossed with 'Royal Gala' and A20R02T032 were used. The stratification protocol was the same as that in NZ. The 2-week old seedlings were transplanted into 8 × 8 × 10 cm pots and grown in an automated ebb and flow fertigation system (Lee et al. 2000) until the end of the experiment.

Venturia inaequalis isolates and phenotyping

For the NZ studies, seven isolates of *V. inaequalis* that were incompatible with the resistance genes in each host, were selected: five of race 1 and two of race 8 (Table 1). Conidial suspensions of monospore cultures for the 'Royal Gala' × TSR34T15 family were prepared from conidia harvested from cultures grown on cellophane overlaid on potato dextrose agar (Parker et al. 1995) and applied at 6–20 × 10^4 conidia ml^{-1}. For the 'Granny Smith' × TSR33T239 family, conidia were harvested from cultures on malt extract agar and applied at 8 × 10^4 conidia ml^{-1}. The inoculum of isolate J222 used in year 2002 on all of the other families was prepared from infected leaves collected from seedlings inoculated with conidia produced in cellophane cultures of the monospore isolate on PDA during the previous year and applied at 2 × 10^5 conidia ml^{-1}. The infected leaves were dried at ambient air temperature and stored at −20 °C. All inoculations were performed by spray-inoculating the seedlings until run-off at the 3–5-leaf stage, followed by incubation under optimal conditions for infection (Gardiner et al. 1996). The phenotyping of the seedlings was performed in the third week after inoculation using the scale developed by Chevalier et al. (1991) with a separate class added for SN.

For the NL study, the host-pathogen interactions of isolates EU-B05, 1639, EU-D42, NZ188B.2 and EU-NL24 (Table 1) were studied on the A68R03T057 and TSR34T15 progenies. The virulence of isolate 1639 towards the *Vh2* gene of host TSR34T15 had been confirmed in a

Table 1. The *Venturia inaequalis* isolates used to study differential interactions in apple

Isolate	Origin	Location	Year	Race
NZ110.1	'Baujade' fruit	Nelson, New Zealand	1996	1
NZ110.3	'Baujade' fruit	Nelson, New Zealand	1996	1
NZ139.1	'Granny Smith' leaf	Auckland, New Zealand	1996	1
J222	Leaf of unknown cultivar	Nelson, New Zealand	1996	1
J243	'Granny Smith' leaf	Auckland, New Zealand	1997	1
EU-B05	'Schone van Boskoop'	Gembloux, Belgium	1998	1
1639	TSR34T15 fruit	Beaucouzé, France	2001	2
EU-D42	'Prima' leaf	Ahrensburg, Germany	1999	6
NZ188B.2	'Royal Gala' × *M. sieversii* GMAL4190 seedling leaf	Havelock North, New Zealand	2000	8
NZ193B.2	'Royal Gala' × *M. sieversii* GMAL3631 seedling leaf	Havelock North, New Zealand	2000	8
EU-NL24[a]	'Prima'	Eekelrade, Netherlands		

[a] Isolate EU-NL24 is virulent to the *Vf*, *Vfh*, and *Vg* genes (Calenge et al. 2004).

preliminary study involving the inoculation of the scab differential host range (data not presented). Inoculum at a concentration of 2×10^4 conidia ml^{-1} was prepared from previously infected seedling leaves. The seedlings were inoculated by placing 100 μl of inoculum into small inoculation chambers, which were made from microtube lids with a 7 mm hole drilled in the top and glued onto hair clips. The chambers were clipped onto the youngest expanded leaf of actively growing shoots for the period of infection establishment. The plants were incubated in dark plastic tents at ambient temperature with the ventilation temperature of the glasshouse set at 20 °C and 100% humidity. The Southern Snap^{TM} × A68R03T057 family was inoculated (number of replicates in parentheses) with EU-B05 (1×), 1639 (2×), EU-D42 (1×), NZ188B.2 (2×), and EU-NL24 (1×); the 'Royal Gala' × TSR34T15 family one time each with EU-B05, 1639, and NZ188B.2; and the A20R02T032 × TSR24T15 family with EU-B05 (1×) and 1639 (2×). After 48 h, the plants were taken out of the tents and the inoculation chambers were removed. The plants were maintained in a glasshouse with >90% relative humidity and ambient temperature. Symptoms were assessed after 2 and 4 weeks and classed as susceptible (S), chlorotic (Chl) very similar to those conditioned by the *Vf* gene (Chevalier et al. 1991), or SN.

DNA extraction, primers, PCR amplification, and map construction

Genetic marker studies were performed on three families: the Southern Snap^{TM} × A68R03T057 for *Vr*-A, the NZ 'Royal Gala' × TSR34T15 for *Vh2*, and a 'Royal Gala' × TSR33T239 cross made in 1996 for *Vh4*, the same family that was used to identify the RAPD markers in the earlier bulked segregant analysis study (Bus et al. 2000). The latter family was phenotyped in 1997 in a glasshouse screen with a mixture of New Zealand race 1 isolates, showing a R:S = 112:89 segregation, which did not differ significantly from R:S = 1:1 ($p(\chi^2 > 2.63) \sim 0.10$). The leaves of the seedlings from the families for the DNA extraction (Gardiner et al. 1996) were sampled before the plants were inoculated.

The polymerase chain reaction (PCR) primers examined and the annealing temperatures used are given in Table 2. The RAPD markers OPL19 and OPB10 (Operon Technologies Inc., Alameda, CA), which had been found to be nearest to the resistance genes of interest in the preliminary study (Bus et al. 2000), were converted to sequence characterised amplified region (SCAR) markers according to Paran and Michelmore (1993). Other markers included in this study were S22SCAR (Hemmat et al. 2002), Z13SCAR (C. Gessler, pers. comm.), and microsatellite markers CH02b10, CH02c02a, CH05e03 and CH03d10 for LG2 (Liebhard et al. 2002).

The PCR mixture for all but one of the SCAR primers was a 15 μl reaction mix containing 1.5 ng of apple genomic DNA, 20 mM Tris–HCl (pH 8.4) and 50 mM KCl buffer, 1.3 mM $MgCl_2$, 1% formamide in distilled H_2O, 0.1 mM of each dNTP, 0.1 μM of each primer, and 0.44 units of Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA), overlaid with 15 μl of

Table 2. SSR and SCAR primers used to generate markers linked to the *Vh2* and *Vh4* apple scab resistance genes in this study

Primer	Sequence	Annealing temperature[a] (°C)
CH02b10SSR[b]	For 5′-CAAGGAAATCATCAAAGATTCAAG-3’ Rev 5′-*GTTT*CAAGTGGCTTCGGATAGTTG-3′	Ta 55
CH02c02aSSR	For 5′-CTTCAAGTTCAGCATCAAGACAA-3′ Rev 5′-TAGGGCACACTTGCTGGTC- 3′	Ta 55
CH03d01SSR	For 5′-CGCACCACAAATCCAACTC-3′ Rev 5′-AGAGTCAGAAGCACAGCCTC- 3′	Ta 55
CH05e03SSR	For 5′-CGAATATTTTCACTCTGACTGGG-3′ Rev 5′-CAAGTTGTTGTACTGCTCCGAC- 3′	Ta 55
OPB10SCAR	For 5′-CTGGGACGAATGTACTCTTTGCTG C-3′ Rev 5′-GTGTCTTCGGATTTCCACTTGATG T-3′	Td 70–60
OPL19SCAR	For 5′-ACCTGCACTACAATCTTCACTAATC-3′ Rev 5′-GACTCGTTTCCACTGAGGATATTTG- 3′	Ta 55
S22SCAR	For 5′-GTCGTGGAAGAGGACCGA-3′ Rev 5′-GTCGTGGAAATCCTCGTGAG- 3′	Td 65–55
Z13SCAR	For 5′-CCCTAGCATGCCATAAAACC-3′ Rev 5′-CCCAGTGGAATATTTCGAGG- 3′	Td 65–55

[a] Td = touchdown annealing temperature range, Ta = set annealing temperature.
[b] The forward primer for this SSR marker was labelled with the fluorescent dye 6-Fam and the reverse primer had a 4 nucleotide PIGtail (Brownstein et al. 1996) added to the 5′ end (italicised nucleotides) to drive the PCR towards complete plus A addition.

paraffin oil. For the OPB10SCAR, the reaction mixture was the same, except for the formamide concentration, which was increased to 2%. DNA was amplified in a Hybaid PCR express thermal cycler with the following programme for OPL19SCAR: 1 cycle of 2 min 45 s at 94 °C; 40 cycles of 55 s at 94 °C, 55 s at 55 °C, 1 min 39 s at 72 °C; and a final extension cycle of 10 min at 72 °C. For the primers requiring a touchdown annealing temperature range (Table 2), the programme was as follows: 1 cycle of 2 min 45 s at 94 °C; 20 cycles of 55 s at 94 °C, 55 s at the touchdown annealing temperature specific to the primer pair (dropping 0.5 °C/cycle), 1 min 39 s at 72 °C; 20 cycles of 55 s at 94 °C, 55 s at the lowest touchdown annealing temperature specific to the primer pair, 1 min 39 s at 72 °C; and a final extension cycle of 10 min at 72 °C. The PCR products were analysed on 0.9% gels (50% Ultrapure agarose (USB Corp., Cleveland, OH), 50% Low Range Ultra Agarose Certified™ (Bio-Rad, Richmond, CA)).

The SSR markers were analysed in the same multiplex. Their PCR amplification solution was a 20 μl reaction mix containing 10 ng DNA; 20 mM Tris–HCl (pH 8.4) and 50 mM KCl buffer; 1.5 mM $MgCl_2$; 0.2 mM of dNTP; the same amount of forward and reverse primer specific to the marker: 0.07 μM for CH02b10, 0.13 μM for CH02c02a, 0.06 μM for CH03d01, and 0.08 μM for CH05e03; 8.54 μl sterile distilled H_2O; and 0.3 units of Platinum *Taq* DNA polymerase (Promega Corp., Madison, WI). DNA was amplified in a Techne Genius thermal cycler (Techne Cambridge Ltd, Cambridge, UK) using the following touchdown PCR conditions: 1 cycle of 2 min 30 s at 94 °C; 33 cycles of 30 s at 94 °C, 30 s at 55 °C, and 1 min at 72 °C; and a final extension cycle of 5 min at 72 °C. The PCR products were loaded onto an ABI PRISM™ 377XL sequencer (Applied Biosystems, Foster City, CA) with an internal 50–500 bp size standard. Data were collected with ABI PRISM™ Data Collection software V2.6, and analysed with ABI PRISM™ GeneScan V3.1.2 and ABI PRISM™ Genotyper V2.1 software. The sizes of all the microsatellite marker alleles were standardised to those presented by Liebhard et al. (2002).

The genetic maps around the resistance genes were constructed using the programme JoinMap Version 3.0 (Van Ooijen and Voorrips 2001). Initial genetic linkage analysis was performed using a LOD score of 5.0 to associate loci into linkage groups, while the threshold LOD score for mapping was set at 1.0. In the Southern Snap™ × A68R03T057 population, as the dataset for the CH03d01 marker was incomplete, a LOD score of 2.0 was used to associate the loci detected by this

marker with the remaining loci of the linkage group. The final locus order was determined by extensive proof reading and by minimising the number of double crossovers flanking single loci. Recombination frequencies were converted to map distances using Kosambi's mapping function (Kosambi 1944) and χ^2 tests were used to identify any segregation distortion of parental alleles.

Results

Resistance phenotypes and segregation ratios

The genes *Vh2* and *Vh4* conditioned resistance reactions, which were distinctly different from each other as well as from symptoms on the susceptible seedlings. The resistant seedlings in the progenies derived from host 2 developed typical SN reactions up to several millimeters in diameter. The resistant seedlings from the host 4 progenies exhibited typical HR reactions, although some seedlings also showed larger, chlorotic rather than necrotic resistance reactions due to coalescence of the reactions.

The segregation ratios of the families derived from both host 2 and 4 were not significantly different (at $p = 1\%$) from R:S = 1:1 (Table 3). The segregation ratios of the pooled data for each resistance gene did also not differ significantly ($p > 0.05$) from a R:S ratio of 1:1, although the host 4 families tended to be more heterogeneous (Table 3). The ratios are consistent with the resistance in each host being conditioned by a single major gene. We have named the genes *Vh2* and *Vh4* respectively, with the *V* referring to *V. inaequalis* and the subscripts *h2* and *h4* referring to the respective hosts 2 and 4 that contain these genes.

The presence of the single gene *Vh2* in accession TSR34T15 was confirmed in the NL study, where

Table 3. The segregation data for the families derived from apple scab differentials 2 and 4 after inoculation with conidia from monospore cultures of seven *V-inaequalis* isolates (R = resistant, S = susceptible)

	Year of cross	Year of inoculation	Female parent	Male parent	Isolate	Number of seedlings			R:S = 1:1	
						Total	R	S	χ^2	*p*
TSR34T15 (apple scab differential host 2)										
1	1999	2001	Royal Gala	TSR34T15	NZ110.1	25	13	12	0.04	0.86
					NZ188B.2	23	11	12	0.04	0.86
					NZ193B.2	23	9	14	1.09	0.30
					J243	26	12	14	0.15	0.70
2	2001	2002	Pacific Queen™	TSR34T15	J222	106	60	46	1.85	0.19
3	2001	2002	A20R01T289	TSR34T15	J222	86	41	45	0.19	0.66
4	2001	2002	A20R02T032	TSR34T15	J222	101	48	53	0.25	0.65
5	2001	2002	A22R11T137	TSR34T15	J222	68	33	35	0.06	0.80
6	2001	2002	Pacific Beauty™	AA848	J222	70	36	34	0.06	0.80
7	2001	2002	Pacific Beauty™	AA962	J222	71	33	38	0.35	0.55
				Pooled	df = 1	599	296	303	0.08	0.77
				Heterogeneity	df = 9				3.99	0.91
TSR33T239 (apple scab differential host 4)										
8	1997	1998	Granny Smith	TSR33T239	NZ110.1	80	47	33	2.45	0.12
					NZ110.3	79	42	37	0.32	0.60
					NZ139.1	70	34	36	0.06	0.80
					J222	81	36	45	1.00	0.32
					J243	69	25	44	5.23	0.02
9	1999	2002	Royal Gala	TSR33T239	J222	49	25	24	0.02	0.90
10	2001	2002	Pacific Queen™	TSR33T239	J222	84	39	45	0.43	0.50
11	2001	2002	A19R03T127	TSR33T239	J222	103	49	54	0.24	0.65
12	2001	2002	A22R11T137	TSR33T239	J222	73	32	41	1.11	0.30
13	2001	2002	A22R16T173	TSR33T239	J222	92	36	56	4.35	0.04
				Pooled	df = 1	780	365	415	3.21	0.14
				Heterogeneity	df = 9				11.99	0.22

the seedlings were either resistant to EU-B05 and NZ188B.2, or susceptible to both isolates. The segregation ratios did not significantly differ from R:S = 1:1 in either host 2 progeny: R:S = 52:53, $p(\chi^2 > 0.010) \sim 0.92$ in the cross with 'Royal Gala' and R:S = 49:57, $p(\chi^2 > 0.602) \sim 0.40$ in the cross with A20R02T032. These two isolates as well as EU-D62 and EU-NL24 induced the same SN (or sometimes only necrosis due to the high spore concentration of the droplets often preventing the development of the typical SN symptoms) reactions in the resistant progeny of the Southern Snap^{TM} × A68R03T057 family as in the *Vh2* families. The seedling segregation ratio of R:S = 56:55 for the *Vr*-A gene in this family (Table 4) did not significantly differ from a R:S = 1:1 ratio ($p(\chi^2 > 0.009) \sim 0.92$). In contrast, isolate 1639 produced R:S = 0:1 ratios in the *Vr*-A as well as the two *Vh2* families, i.e. it was compatible with both genes and demonstrates that the gene in A68R03T057 is not *Vr*-DW, but is in fact *Vh2*. Isolate 1639 also was the only isolate that was incompatible with a second single gene segregating in the Southern Snap^{TM} × A68R03T057 family, which conditioned Chl reactions in the progeny carrying the gene. The segregation ratio of R:S = 53:58 (Table 4) for this new gene, which we temporarily have named *VT57*, was not significantly different from R:S = 1:1 ($p(\chi^2 > 0.225) \sim 0.60$). The *Vr*-A and *VT57* genes were linked at a distance of 10.8 recombination units, and since *VT57* was linked in repulsion with *Vr*-A, it originated from one of the grandparents of A68R03T057, 'Golden Delicious' or 'Red Dougherty'. In the absence of marker data for 'Red Dougherty', it could not be established with certainty from which cultivar the gene originated. Neither *VT57* nor any other genes except for *Vh2* were segregating in the TSR34T15 families.

Table 4. Segregation of two scab resistance genes in a Southern Snap^{TM} × A68R03T057 family

Vr-A	*VT57*		Total
	R	S	
R	5	51	56
S	48	7	55
Total	53	58	111

The segregation of the *Vr*-A gene is based on the incompatible interaction with isolates EU-B05, EU-D42, NZ188B.2 and EU-NL24, and the segregation of the *VT57* gene is based on the incompatible interaction with isolate 1639.

Genetic markers and linkage maps

Both the *Vh2* and *Vr*-A genes co-segregated with the microsatellite marker CH02b10 in their respective families, as had *Vr*-A in the study by Hemmat et al. (2002). At a length of 124 bp (Figure 2), the allele linked to the resistance gene in our TSR34T15 family was longer than the 122 bp reported in their study. However, DNA of Russian apple 12740-7A provided by M. Hemmat exhibited the same 124 bp band under our analytical conditions. In our study, we used a 4 nucleotide PIGtail (Table 2) to drive non-template A addition to completion, hence this difference will be partly due to the PIGtail addition and the possibility that the PCR product will be one bp longer due to the non-template addition (Brownstein et al. 1996). Calibration of the marker with the reference cultivar 'Prima' showed a consistent difference of 3 bp in the alleles in our study compared to those found by Liebhard et al. (2002). When standardised to the allele sizes of Liebhard et al. (2002), the allele CH02b10 linked to the *Vh2* gene therefore is 121 bp. Since the measurement of the size of the alleles was determined on agarose by Hemmat et al. (2002) as compared to a DNA sequencer in our study, the limit to accurate measurement of at least one of these methods is likely to be greater than the 1 bp difference. Hence, it also is likely that they are actually the same allele and therefore have the same size. The CH02b10 marker also mapped at very similar distances above *Vh2* (8 cM) and *Vr*-A (10 cM) in our study (Figure 3) as it did to *Vr*-A (7 cM) in the study by Hemmat et al. (2002). Linkage of two other microsatellite markers (CH05e03 and CH03d01) to both genes in our families confirmed that they map to the distal end of LG2 of apple (Figure 3). The maps, however, differed in the position of the resistance genes relative to the CH05e03 and OPL19SCAR markers, as they mapped above *Vr*-A on the A68R03T057 map, and below *Vh2* on the TSR34T15 map. The presence of the OPB18SCAR marker in host 2 (Hemmat et al. 2002, coded GMAL 2411 in their study) was confirmed, but the

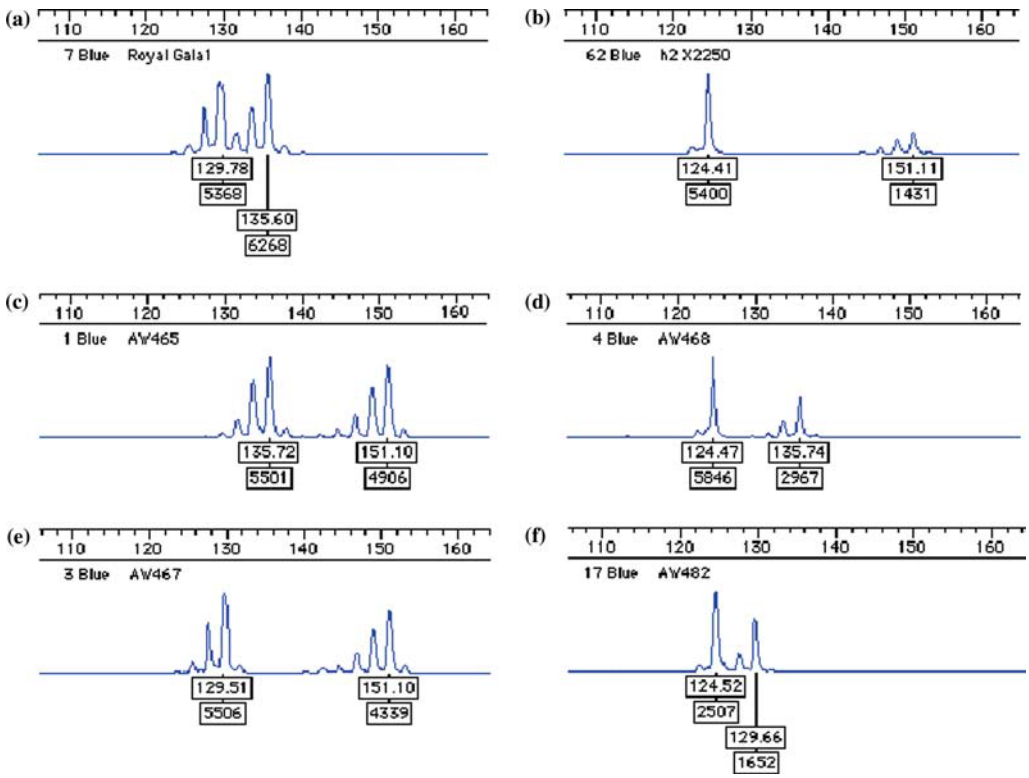

Figure 2. Microsatellite genotype with the marker CH02b10 of the susceptible parent 'Royal Gala' (A) and the resistant parent TSR34T15 (B), and 4 progeny showing the 4 possible allele combinations (panels C–F). Progenies D and F are resistant and C and E are susceptible.

marker did not segregate in the 'Royal Gala' × TSR34T15 progeny.

Linkage of the S22SCAR marker to an apple scab resistance gene conditioning an HR (Hemmat et al. 2002) was also confirmed in host 4. The marker was amplified in TSR33T239 (coded W7AR44T20 in the study by Hemmat et al. (2002), but erroneously identified as host 2) and its resistant progeny. However, at 4 cM, the marker is somewhat closer to the *Vh4* gene in our 'Royal Gala' × TSR33T239 family, compared to its map distance to the *Vx* gene in the 'Empire' × Russian apple R12740-7A family (Hemmat et al. 2002). The OPB10 > 2000bpSCAR marker mapped at a considerable distance from the *Vh4* gene (Figure 3). However, mapping with microsatellite markers unambiguously assigned the gene to the proximal end of LG2 of apple near CH02c02a (Figure 3). A multiplex of four microsatellite markers from LG10 (CH02b07, CH02a10, CH02c11 and Col (Liebhard et al. 2002) had been tested first, but none of the markers segregated with the resistance gene (data not presented).

Discussion

Scab resistance genes and phenotypes

The resistance symptoms present in the progenies of the individual hosts 2 and 4 are consistent with earlier findings that Russian apple R12740-7A contains a gene conditioning HR reactions, and one that conditions SN reactions (Hemmat et al. 2002). Since both hosts 2 and 4 were derived from Russian apple, we confirm that the *Vh2* gene in host 2 is the same as *Vr*-A, and that the *Vh4* gene in host 4 is the same gene as *Vx* based on both resistance symptoms and marker data. As no other major scab resistance genes were found to segregate in the TSR34T15 and TSR33T239 families with any of the isolates tested under glasshouse conditions, we conclude that these two accessions

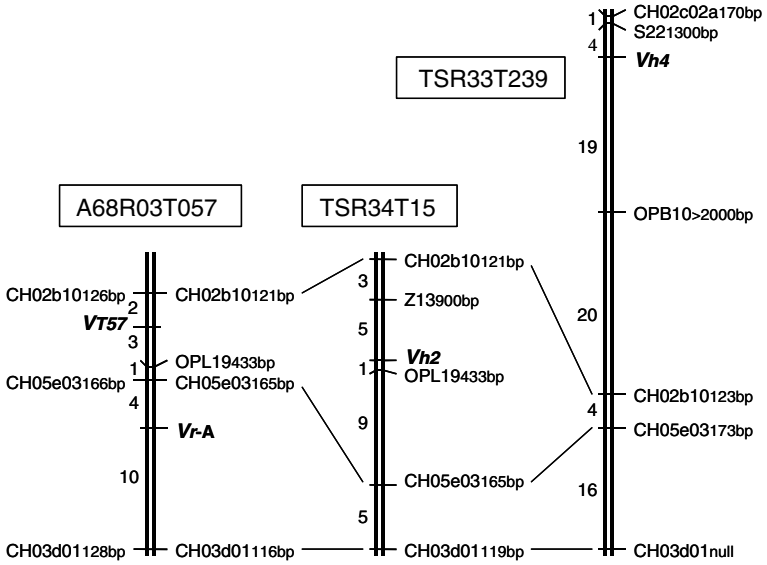

Figure 3. The genetic maps of the genomic regions around the scab resistance genes *Vr*-A and *VT57* in A68R03T057, *Vh2* in TSR34T15, and *Vh4* in TSR33T239 on linkage group 2 of apple. The markers starting with CH are microsatellite markers (Liebhard et al. 2002) and all other markers are SCARs.

carry a single gene each. Each host, therefore, inherited only one gene from their parents PRI 45-39 (host 2) and PRI 27-330 (hosts 4) (Figure 1), respectively, which both had been shown to carry more than one resistance gene (Dayton et al. 1953).

The Vh4/Vx gene

A major finding of this study is that, while the linkage of S221300bpSCAR to the HR conditioning gene (Hemmat et al. 2002) was confirmed, the gene and marker mapped to LG2 in our 'Royal Gala' × TSR33T239 family. These findings are in contrast with an earlier report mapping it to LG6 of 'Rome Beauty' × 'White Angel' (RB × WA) (Hemmat et al. 2003b), which is the equivalent of LG10 of the European apple map (Maliepaard et al. 1998). It should be noted that the mapping of the gene onto LG10 in that study was based on a single marker shared between the European and RB × WA maps, and was therefore only tentative. However, this information has already been used to justify naming a putatitively new gene, *Vr1*, which maps onto LG2 (Boudichevskaia et al. 2004), when in fact it maps to the same region as *Vh4* and therefore most likely is the same gene.

The S221300bpSCAR marker mapped much closer to the gene in our 'Royal Gala' × TSR33T239 family than in the 'Empire' × Russian apple R12740-7A family (Hemmat et al. 2002). This could be due to the fact that maps made in different genetic backgrounds often differ in absolute map distances. Another factor could be the greater certainty in the phenotyping of the progenies segregating for a single gene in our study, as compared to a multiplex of at least two major genes segregating in the Russian apple family studied by Hemmat et al. (2002). Their phenotyping may have been complicated further by the use of inoculum consisting of a number of races, including those that could overcome the individual resistance genes in Russian apple (Hemmat et al. 2002). Due to possible epistatic interactions between resistance genes, which cannot easily be predicted, such an approach leads to a degree of uncertainty when relating the observed phenotype to a particular gene.

Based on its pedigree, HR phenotype, and linkage to S221300bpSCAR and CH02c02a, we conclude that the *Vr1* gene segregating in a progeny of 'Regia' (Boudichevskaia et al. 2004), also a descendant of Russian apple, is identical to *Vh4*. Another HR conditioning gene, *Vr2*, is also linked to CH02c02a (Patocchi et al. 2004), but uncertainty

about the origin of the Russian apple accession used, the low number of seedlings with distinctive HR, and the absence of S221300bpSCAR suggest that while *Vr2* maps to the same region at the proximal end of LG2 as *Vh4*, it is either a different gene or it may be *Vh4* and simply has lost a closely associated marker allele.

The Vh2/Vr gene

Linkage of the microsatellite markers CH02b10, CH05e03 and CH03d01 to the SN conditioning *Vh2* gene clearly maps this scab resistance gene to the distal end of LG2 of the apple genetic map (Liebhard et al. 2002). The genetic marker analysis showed that the 121 bp of the CH02b10 marker is linked in coupling to the race-specific *Vh2* gene, the *Vr*-A gene in the A68R03T057 family, and the SN conditioning gene designated *Vr* (*Vr*-A) by Hemmat et al. (2002) and maps at very similar distances to the genes. This, together with the phenotypic data from inoculation with isolate 1639 capable of overcoming the SN conditioning genes in our *Vr*-A and *Vh2* families, indicates that they are the same gene. This also means that the SN conditioning gene cannot be the gene originally named *Vr* (*Vr*-DW), since it was attributed to a race-nonspecific scab resistance gene (Dayton and Williams 1968; Hough et al. 1970; Crosby et al. 1992).

As mentioned above, the name *Vr*-A was assigned to the gene conditioning these distinctive SN reactions in a progeny of the F2 Russian apple descendant NY58516-5 (Figure 4) to discern it from another resistance gene in Russian apple conditioning a Chl phenotype not previously described (Aldwinckle et al. 1976), even though it had been reported earlier that the resistance in other Russian apple descendants carrying a gene conditioning SN symptoms, were overcome by a race of the scab fungus (Williams and Shay 1957). This implies that Aldwinckle et al. (1976) recognised that only one of the resistance genes from Russian apple could be designated *Vr*, and that there still was uncertainty about which one of these was the same as the gene designated *Vr* by Dayton and Williams (1968). However, there are indications in the latter paper that at least two of the four non-differential accessions used in this study carried two scab resistance genes instead of one. Epistasis of the putative race-nonspecific gene to a possibly unrecognised gene conditioning Chl explains the anomalies found in the study on independently segregating scab resistance genes by Dayton and Williams (1968). Crossed with each other, progeny with the pyramided resistance genes crossed in turn with susceptible cultivars, are expected to produce R:S = 3:1 ratios. However, two F2 derivatives of Russian apple (PRI 401-1 and PRI 462-14 (Figure 4)) crossed with different *Vf* selections, produced F3 progenies (TSR21T164 and TSR25T122, respectively), showing segregations that fitted the R:S = 7:1 ratio when crossed with the susceptible cultivar 'McIntosh' (Table 5). Even though genes mapping to the same LG were involved, as we have shown to be the case with *Vh2* and *Vh4*, the R:S = 7:1 ratios expected of the three gene model generally will result in a good fit if the distance between the genes is 43 cM or more. Preliminary data (not presented) indicate that the non-differential accession PRI 2375-5 also carries two resistance genes, one of which is *Vh2*, the gene that was inherited by A68R03T057. Research has been initiated to study the second gene in PRI 2375-5.

Vh2 and *VT57* identified in this study both map to the same region of LG2 as the *Vbj* (Baldi et al. 2004) and *Vh8* genes (Bus et al. 2004) as well as several quantitative trait loci (QTLs) for scab resistance (Calenge et al. 2004). The *VT57* gene was compatible with isolates EU-D42 and EU-NL24, which indicates that it is different from the QTLs from 'Discovery' and TN10-8 that have been mapped to the LG2 region with the aid of these two isolates (Calenge et al. 2004). The presence of another gene cluster involving *Va* and *Vb* (Hemmat et al. 2003a), *Vf* and QTLs on LG1, and several QTL regions elsewhere on the apple genome (Durel et al. 2003; Liebhard et al. 2003; Calenge et al. 2004) is in line with the identification of resistance gene clusters in many other crops (Michelmore and Meyers 1998).

Conclusion

Our study is the first to date on the symptoms and genetics of the resistances in the differential hosts derived from Russian apple. Studying these hosts instead of Russian apple R12740-7A itself had the advantage that the individual genes in these hosts can be dissected independently. In this study, we have confirmed that the resistances in the Russian apple R12740-7A derived hosts 2 and 4 are

Table 5. The segregation data of the test-crosses (with the scab susceptible cultivar 'McIntosh') of two non-differential F2 progeny derived from Russian apple 12740-7A crossed with sources of the independently segregating *Vf* gene

Progeny number	F2 progeny of Russian	*Vf* source	Number of seedlings			R:S = 3:1		R:S = 7:1	
			Resistant	Susceptible	Total	χ^2	*p*	χ^2	*p*
TSR21T164	PRI 401-1	M.A. 4	222	31	253	21.87	< 0.01	0.01	0.90
TSR25T122	PRI 462-14	Hansen's baccata #1	150	24	174	14.17	< 0.01	0.27	0.63

Adapted from Dayton and Williams (1968). See Figure 4 for the pedigrees.

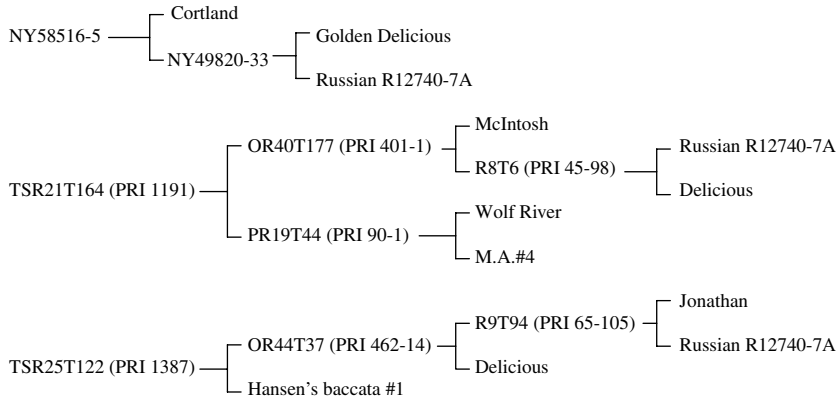

Figure 4. The pedigrees of three descendants of Russian apple referred to in this study. The information was collated from the website of the Purdue-Rutgers-Illinois apple breeding programme (http://www.hort.purdue.edu/newcrop/pri/default.html).

monogenic, with the gene in host 2 conditioning SN symptoms and the one in host 4 conditioning HR symptoms. We have developed detailed maps around each gene and shown that they both map to the opposite ends of LG2 of apple. From the historic data as well as the phenotypic and genetic marker data from this study, we conclude that the *Vh2* and the *Vh4* genes in hosts 2 and 4 are the genes from Russian apple conditioning SN and HR responses, respectively, (named *Vr*-A and *Vx*, respectively, by Hemmat et al. (2002)). A review of the literature surrounding the naming of the *Vr* gene, combined with current knowledge suggests that the gene initially named *Vr*-DW (Dayton and Williams 1968), is not the same gene named *Vr*-A by Alwinckle et al. (1976) and Hemmat et al. (2002). Further research will be required to elucidate the scab resistance gene complex in Russian apple R12740-7A, but the gene conditioning Chl resistance reactions found by Aldwinckle et al. (1976) is a likely candidate to be recognised as the original race-nonspecific scab resistance gene in this host. In order to clarify which genes are being referred to, we therefore suggest retaining our initial designation of *Vh2* and *Vh4* (Bus et al. 2000) for the genes present in the differential hosts 2 and 4, respectively, and retaining the name *Vr* for the third scab resistance gene in Russian apple R12740-7A. This would comply with the well-established convention that the first naming takes precedence over subsequent re-namings. The confusion over the naming of genes illustrates, however, that it may be prudent to establish a new naming system for all scab resistance genes that is more in line with systems adopted in other plants, for example, to a system based on numbers only, in which the scab differential host and the *V. inaequalis* race that can overcome the resistance gene it carries, are assigned the same number.

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