

A. Patocchi · B. Bigler · B. Koller · M. Kellerhals ·  
C. Gessler

## ***Vr*<sub>2</sub>: a new apple scab resistance gene**

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**Abstract** Reports from several European countries of the breakdown of the *Vf* resistance, the most frequently used source of resistance in breeding programs against apple scab, emphasize the urgency of diversifying the basis of apple scab resistance and pyramiding different apple scab resistances with the use of their associated molecular markers. GMAL 2473 is an apple scab resistant selection thought to carry the resistance gene *Vr*. We report the identification by BSA of three AFLP markers and one RAPD marker associated with the GMAL 2473 resistance gene. SSRs associated with the resistance gene were found by (1) identifying the linkage group carrying the apple scab resistance and (2) testing the SSRs previously mapped in the same region. One such SSR, CH02c02a, mapped on linkage group 2, co-segregates with the resistance gene. GMAL 2473 was tested with molecular markers associated with other apple scab resistance genes, and accessions carrying known apple scab resistance genes were tested with the SSR linked to the resistance gene found in GMAL 2473. The results indicate that GMAL 2473 does not carry *Vr*, and that a new apple scab resistance gene, named *Vr*<sub>2</sub>, has been identified.

### **Introduction**

Apple scab, caused by the fungal pathogen *Venturia inaequalis* (Cke), is the most important apple disease, and is present in all apple growing regions. Up to 15

treatments with fungicides are necessary each year to prevent economic losses due to this pathogen. The increased ecological sensitivity of growers and requests by consumers for organically produced apples have stimulated breeders to select apple scab resistant cultivars. The most commonly used apple scab resistance gene is *Vf*, derived from *Malus floribunda* 821. Currently, *Vf* resistance is present in more than 70 scab-resistant cultivars (Janick et al. 1996). The popularity of *Vf* has encouraged many researchers to study this resistance. This has led to the discovery of several molecular markers linked to *Vf* (Koller et al. 1994; Gianfranceschi et al. 1996; Tartarini 1996; Tartarini et al. 1999). These markers were later used to begin the positional cloning of the gene (Vinatzer et al. 1998; Patocchi et al. 1999a, b; Vinatzer et al. 2001) resulting in the cloning of the first apple scab resistance gene (Barbieri et al. 2003, Belfanti et al. 2004).

The appearance of the apple scab races 6 and 7, which are able to overcome the *Vf* gene (Parisi et al. 1993; Bénaouf and Parisi 2000), in several European countries has prompted breeders to broaden the genetic basis of resistance against scab. Development of molecular markers associated with the corresponding resistances is thus required. Durable resistance may be achieved by pyramiding several major resistance genes in the same background. This can be done by crossing cultivars carrying different sources of resistance and using molecular markers associated with them to select the plants with the desired allele combination (marker assisted selection, MAS).

Five loci conferring qualitative apple scab resistance, in addition to *Vf*, have been identified. They mainly originate from small-fruited Asiatic *Malus* species: *Vm* from *M. micromalus*, *Vr* from *M. pumila* R12740-7A (also called “Russian seedling”), *Vbj* from *M. baccata jackii*, *Vb* from Hansen’s Baccata No. 2 and *Va* from PI 172623 (Williams and Kuc 1969; Lespinasse 1989). All except *Va* have already been introduced into breeding lines, making them available for breeding purposes (Liebhard et al. 2003). However, molecular markers are available for *Vf*, *Vm* (Cheng et al. 1998), *Vbj* (Gygax 2004), *Vr* (Hemmat et

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A. Patocchi (✉) · B. Bigler · B. Koller · C. Gessler  
Plant Pathology Group, Institute of Plant Sciences, Swiss  
Federal Institute of Technology,  
8092 Zürich, Switzerland  
e-mail: andrea.patocchi@ipw.agrl.ethz.ch  
Tel.: +41-1-6325789  
Fax: +41-1-6321572

M. Kellerhals  
Agroscope FAW Wädenswil,  
8820 Wädenswil, Switzerland

al. 2002) and for a second resistance gene, *V<sub>x</sub>*, recently identified in Russian seedling (Hemmat et al. 2002). *V<sub>r</sub>*, *V<sub>bj</sub>*, *V<sub>m</sub>* and *V<sub>f</sub>* are therefore ready to be pyramided in all desired combinations. For the remaining apple scab resistance genes, either advanced selections or molecular markers must first be developed. However, as demonstrated by this study, many more unknown qualitative apple scab resistances, not overcome by the pathogen, are still present in the apple genetic pool and can be used to broaden the genetic basis for apple scab resistance.

In this paper, we report the identification of a qualitative apple scab resistance gene in the accession GMAL 2473, named *V<sub>r2</sub>*, and the development of molecular markers associated with this gene.

## Materials and methods

### Plant material and inoculation

The cross between the apple scab resistant selection GMAL 2473 (PI 589835, from the Geneva National Germplasm Repository) and Idared was performed at the Swiss Federal Station, Wädenswil in 1999. A total of 377 progeny plants were inoculated with local mixed apple scab inoculum as described by Gianfranceschi et al. (1996). Disease symptoms were scored following King et al. (1998): class 0, no symptoms; class 1, pinpoint pits; class 2, chlorotic lesions, possibly small necrotic spots; class 3, chlorotic and necrotic spots; class 4, chlorotic and necrotic spots, presence of sporulation; class 5, sporulation. A subset of progeny plants were inoculated a second time to verify their classification.

### DNA extraction

DNA was extracted following Koller et al. (2000) with the following modifications: leaf samples were lyophilized and the RNA was digested by adding RNase A (Roche) to the lysis buffer. The DNA pellets were washed twice with 70% ethanol, dried and re-suspended in 50 µl double-distilled H<sub>2</sub>O. The DNA concentrations were estimated by gel electrophoresis.

### Bulked segregant analysis

Bulked segregant analysis (BSA) was performed following Michelmore et al. (1991). To exclude possible contaminating outcrossed progeny plants, all plants chosen for the bulks were first tested with five SSR markers; CH04e03, CH01f02, CH01f03b, CH01h02 and CH05c07 (Liebhard et al. 2002a), as described in Gianfranceschi et al. (1998). Plants showing alleles not present in the parents were excluded. For bulk assembly, only those plants that did not differ in classification between the two scab inoculation trials were chosen. Five bulks, each composed of DNA (20 ng/µl) from ten plants, were assembled. Bulk

1 was composed of DNA from plants showing class 1 symptoms. Bulks 2a, 2b, 5a and 5b were generated by mixing DNA from class 2 and class 5 progeny plants respectively. For RAPD testing, the bulks were diluted to 1 ng/µl, while for AFLP analysis the bulks were used at a concentration of 20 ng/µl.

### AFLP and RAPD analyses

AFLP technology, as described by Liebhard et al. (2002b), was applied to scan the five bulks and the two parents with 168 primer combinations (14 *EcoRI* primers, EA31-EA44 and 12 *MseI* primers, MA31-MA42, GibcoBRL Life Technologies, USA). RAPD technology, as described by Koller et al. (1994), was used to identify polymorphisms between the bulks as well as between the two parents. Primers OPA1 to OPZ20 (Qiagen-Operon) excluding OPC10, OPH7, OPO1-O20, OPP1-P20, OPU1-U6 and OPU13-20 were tested. Primers generating polymorphic bands between the resistant bulks (1, 2a and 2b) and the susceptible bulks (5a and 5b) as well as between the parents were tested on 89 plants (50 resistant and 39 susceptible).

### Linkage group identification

Seven plants classified in the apple scab resistance class 2 (resistant), six plants classified in the apple scab resistance class 5 (susceptible) and the two parents of the cross (GMAL 2473 and Idared), were analyzed according to Liebhard et al. (2002b) with the following 33 SSRs (two per linkage group, Liebhard et al. 2002b): CH03g12-1/3, CH05g08-1, CH02c02a-2, CH05e03-2, CH03g07-3, CH01h11a-4, CH02c02b-4, CH05e06-5, CH02a08-5, CH03d07-6, CH03d12-6, CH02a04-7, CH04e05-7, CH01c06-8, CH01h10-8, CH01f03b-9, CH01h02-9, CH02b03-10, CH02c11-10, CH03d02-11, CH04g07-11, CH04g04-12, CH01f02-12, CH05h05-13, CH03h03-13, CH01g05-14, CH04c07-14, CH01d08-15, CH02d11-15, CH05e04-16, CH04f10-16, CH05g03-17 and CH05d08-17. The numbers separated from the SSR name by a hyphen indicate the linkage group.

### Linkage analysis

A linkage map was generated using the software JoinMap version 2.0 (Stam and Van Ooijen 1995) in connection with JMDesk 3.6 (<http://www.ecogenics.ch/software-e.html>).

## Results

### Resistance screening

To our knowledge, the genetic basis of the apple scab resistance of GMAL 2473 has never been studied before. Therefore it was necessary to verify the number of genes conferring this resistance. The progeny plants of the cross GMAL 2473 × Idared were inoculated with apple scab conidia in a glasshouse and 10–12 days later were classified into six resistance/susceptibility classes based on symptoms (Table 1). No clear segregation ratio was observed. The percentage of susceptible progeny plants, where the threshold between resistant and susceptible plants was placed between classes 3 and 4 or 4 and 5 (threshold 3/4 or 4/5), was 42.4 and 35%, respectively.

The hypotheses of the presence of either one single or two major genes were tested. The possibility of two major dominant apple scab resistance genes segregating in GMAL 2473 was immediately rejected. The ratio between resistant and susceptible progeny plants, with both thresholds, differed significantly from the 3:1 ratio expected ( $\chi^2=61.15$ ,  $\chi^2=21.16$ ). However, the presence of only a single major resistance gene could not be statistically proven. With both thresholds, the ratios differed significantly from the 1:1 ratio expected ( $\chi^2=8.61$  with threshold 3/4 and  $\chi^2=33.87$  with threshold 4/5).

### Bulked segregant analysis

Bulks for BSA were assembled by pooling DNA from plants exhibiting the most extreme resistance reactions; resistant plants (classes 1 and 2) and susceptible plants (class 5). The DNA of plants showing pinpoint pits (class 1) or chlorotic-necrotic lesions (class 2) were kept in separate bulks.

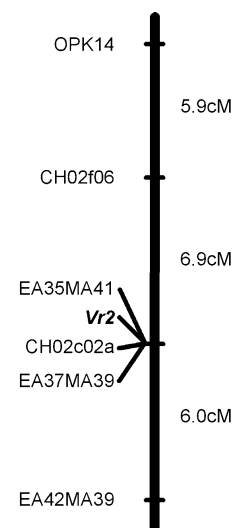
Ten out of 464 RAPD primers tested showed a polymorphism between the resistant and the susceptible bulks, as well as between the parents. No differences were noted between the bulks of plants classified as resistance class 1 and the bulks of plants classified as resistance class 2. Six polymorphic bands were in coupling with the resistance, while four were in repulsion. Only a single marker, OPK14<sub>750</sub>, demonstrated an association with resistance below 20% (12% recombination) after testing with a subset of 89 progeny plants. OPK14<sub>750</sub> is in coupling with the resistance, maps at 12.8 cM from the resistance, and the size of the polymorphic band is about 750 bp.

With the AFLP technique, 13 out of 168 primer combinations tested produced polymorphic bands between the resistant and the susceptible bulks, as well as between the parents. As was the case for the RAPD polymorphic bands, no differences among the resistant bulks were observed. After testing the subset of 89 progeny plants, only three markers, EA35MA41<sub>262</sub>, EA37MA39<sub>188</sub> and EA42MA39<sub>500</sub>, showed an association with resistance. EA35MA41<sub>262</sub> and EA37MA39<sub>188</sub> mapped at the same position as the resistance gene, while EA42MA39<sub>500</sub> mapped 6 cM from it. The sizes of the polymorphic fragments of EA35MA41<sub>262</sub>, EA37MA39<sub>188</sub> and EA42MA39<sub>500</sub> were 262, 188 and about 500 bp respectively.

### Identification of the linkage group carrying the apple scab resistance gene

Thirteen progeny plants and the two parents were analyzed with 33 SSRs. SSR CH02c02a, previously mapped on linkage group 2, showed association with the apple scab resistance gene. CH02f06 another SSR previously mapped on near CH02c02a and CH02c02a linkage group 2, were subsequently tested on the subset of 89 progeny plants to confirm their association with resistance. CH02c02a was mapped at the same position as the two AFLP markers EA35MA41<sub>262</sub> and EA37MA39<sub>188</sub> and the resistance gene. CH02f06 was mapped at 6.9 cM from the resistance gene. We named the apple scab resistance gene identified in GMAL 2473, *Vr2* (Fig. 1). The SSR alleles in coupling with the *Vr2* are 176 and 146 bp long for CH02c02a and CH02f06, respectively.

**Fig. 1** Genetic map of the *Vr2* region, based on data from 89 progeny plants of the cross GMAL 2473 × Idared belonging to the resistance classes 1, 2 and 5. The names of AFLP markers are composed of the *EcoRI* and the *MseI* primer names and the length of the polymorphic fragment



**Table 1** Scab resistance scoring of the 377 progeny plants of the cross GMAL 2473 × Idared. Resistance was scored according to the scale of King et al. (1998)

	Resistance classes					
	0	1	2	3	4	5
Number of progeny plants per class	3	45	129	40	28	132
Percentage of progeny plants per class	0.8	11.9	34.2	10.6	7.4	35.0

## Discussion

### Apple scab resistance of GMAL 2473

The determination of the number of resistance genes present in a cultivar from the distribution of progeny plants in resistance classes relies on proper placement of the threshold between resistant and susceptible classes. For the apple scab resistance *Vf*, this threshold has been placed between classes 4 and 5 (Gardiner et al. 1996). Although artificial, this threshold is biologically reasonable since only plants belonging to class 5 show clear sporulating lesions and no plant resistance reaction (Chevalier et al. 1991). Molecular markers for *Vf* confirmed the correct positioning of this threshold. The same threshold was used for the apple scab resistance *Vbj*, where clear segregation ratios were obtained (Gygax 2004).

This threshold, however, is not ideal for the apple scab resistance carried by GMAL 2473. In fact when applying this threshold, both hypotheses formulated—presence of a single gene or of two major apple scab resistance genes—were statistically rejected. Even with a threshold between classes 3 and 4 both hypotheses were discarded. With this second threshold, however, the hypothesis of the presence of a single gene performed better than the two-gene hypothesis. Most progeny plants were inoculated only once and therefore a certain percentage of the population may have escaped inoculation and been classified as resistant instead of as susceptible. If we assume that 5% of the susceptible plants escaped inoculation, the only statistically verified hypothesis is the presence of a single major resistance gene ( $\chi^2=0.27$ ).

The analysis of 154 unselected progeny plants with the SSR CH02c02a clearly confirm that *Vr<sub>2</sub>* is present in the classes 0–3 and is absent in classes 4 and 5 (Table 2). Class 4 and 5 plants are the only two groups of plants that show sporulation. Class 5 plants exhibit heavy sporulation and do not show any resistance reaction. On the contrary, class 4 plants exhibit only light sporulation and show a resistance reaction (formation of chlorotic and necrotic lesions). The fact that class 4 plants lack *Vr<sub>2</sub>* but show resistance reactions may be explained by the presence of QTLs for apple scab resistance. Moreover, the fact that only the plants lacking *Vr<sub>2</sub>* show sporulation permits us to speculate that *Vr<sub>2</sub>* inhibits sporulation.

*Vr<sub>2</sub>* is a previously unknown apple scab resistance

gene. An exclusion process permits us to conclude that *Vr<sub>2</sub>* is a previously unidentified apple scab resistance gene. The use of molecular markers linked to *Vr<sub>2</sub>* and the other known resistance genes has allowed us to confirm that *Vr<sub>2</sub>* is unique.

The resistance gene *Vf* maps on linkage group 1 (Liebhard et al. 2002b) and *Vr<sub>2</sub>* on linkage group 2. Furthermore, tests on GMAL 2473 with the *Vf* molecular markers AL07 (Tartarini et al. 1999) and HcrVf2 (Vinatzer et al. 2001) were both negative (no amplification of the allele in coupling with the resistance), showing that *Vf* and *Vr<sub>2</sub>* are different. GMAL 2473 was previously tested by Cheng et al. (1998) with the molecular marker OPB12<sub>687</sub> associated with *Vm* and was found to be negative, showing that *Vr<sub>2</sub>* is different from *Vm*.

Hansen Baccata No. 2, the accession carrying the resistance gene *Vb* (Williams and Kuc 1969), has been analyzed with the two SSRs associated with *Vr<sub>2</sub>*, CH02c02a and CH02f06. Neither of the alleles associated with *Vr<sub>2</sub>*, respectively 176 and 146 bp long, were amplified. In Hansen Baccata No. 2, the SSR CH02c02a amplified two fragments of 128 and 158 bp, while CH02f06 amplified two fragments of 130 and 154 bp, demonstrating that *Vr<sub>2</sub>* is different from *Vb*.

*Vbj* is associated with the SSR CH05e03 (Gygax 2004). Liebhard et al. (2002b) showed that CH05e03 maps on linkage group 2; the same linkage group as *Vr<sub>2</sub>*, but at 47.1 cM from the SSR CH02c02a. Moreover, a test of GMAL 2473 with the *Vbj* marker T06<sub>410</sub> SCAR (Gygax 2004) was negative, showing that GMAL 2473 also does not carry *Vbj*. We wanted to analyze accession PI 172623, the accession carrying *Va* (Williams and Kuc 1969) in the same way, but it was not possible for us to obtain genetic material from the publicly available resources.

Hemmat et al. (2002) recently published molecular markers for two apple scab resistance genes of Russian seedling, *Vr* and *Vx*. *Vr* is associated with the SSR CH02b10 and the RAPD marker OPB18<sub>620</sub>, while *Vx* is associated with the SCAR marker S22<sub>1300</sub>. *Vr* and *Vx*, also called *Vh<sub>2</sub>* and *Vh<sub>4</sub>* respectively by Bus et al. (2000), were mapped on two different linkage groups, *Vr* being mapped on linkage group 2 (Liebhard et al. 2002b). *Vr<sub>2</sub>* also maps on linkage group 2, however the genetic distance between the SSRs associated with *Vr<sub>2</sub>* and *Vr* is 42.9 cM on the map of Liebhard et al. (2002b) and therefore excludes the possibility that the two resistance genes are identical,

**Table 2** Comparison between scab resistance scoring and presence of the CH02c02a allele in coupling with *Vr<sub>2</sub>* in 154 unselected progeny plants

	Resistance classes, based on the scale of King et al. (1998)					
	0	1	2	3	4	5
Number of progeny plants per class	2	10	63	10	9	60
Number of progeny plants per class carrying the CH02c02a allele in coupling with <i>Vr<sub>2</sub></i>	2	10	61	9	0	0
Number of progeny plants per class carrying the CH02c02a allele in repulsion with <i>Vr<sub>2</sub></i>	0	0	2	1	9	60

although both of the *Vr* markers amplified the expected alleles for *Vr* from GMAL 2473. The fact that *Vx* was mapped to a different linkage group than *Vr*, and that *Vr* and *Vr*<sub>2</sub> have been mapped to the same linkage group, allows us to exclude the possibility that *Vr*<sub>2</sub> is the same as *Vx*. Moreover GMAL 2473, when tested with S22<sub>1300</sub>, did not amplify the 1,300 bp band expected for the *Vx* marker.

Until recently GMAL 2473 was considered a clone of R12740-7A (Russian seedling). When DNA of Russian seedling originating from another source (kindly provided by Frank Dunemann, BAZ) was tested with the three markers for *Vr* and *Vx*, all three markers amplified alleles of the expected sizes. Comparison of five SSR allele pairs amplified from GMAL 2473 and the second Russian seedling sample confirmed the hypothesis that the two plants differ from each other. The Geneva National Germplasm Repository has confirmed that since 1998, GMAL 2473 (PI 589835) is not considered to be a clone of Russian seedling (alias R12740-7A) and that the correct clone of Russian seedling present at the Geneva National Germplasm Repository is GMAL 1462 (PI 589312). Moreover, GMAL 2473 has been removed from the collection. Plant material of GMAL 2473 can be requested from Agroscope FAW Wädenswil.

To date both GMAL 2473 and GMAL 1462 have been known by the name “Russian seedling”. To avoid confusion we propose renaming GMAL 2473 as “Russian seedling 2” and, consequently, we name the resistance gene inherited from GMAL 2473 *Vr*<sub>2</sub>.

In conclusion, the present work has led to the discovery of a new major apple scab resistance gene and to the development of molecular markers associated with it. Advanced selections carrying *Vr*<sub>2</sub> are now required. *Vr*<sub>2</sub> and its molecular markers can then be used for marker-assisted selection of apple scab resistant cultivars, along with other apple scab resistances for which molecular markers have been developed. This new generation of cultivars will carry several apple scab resistance genes in the same background, reducing the risk of resistance breakdown by the pathogen.

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