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Towards the map-based cloning of *Vf*: fine and physical mapping of the *Vf* Region

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Abstract A map-based cloning scheme is being used to isolate the *Vf* resistance gene of apple against the fungus *Venturia inaequalis*. *Vf* is a major dominant gene that is inherited in a Mendelian manner and influenced by minor genes that modify its activity. The two recently published markers M18 and AL07, bracketing *Vf*, were tested on 1179 progeny plants of three crosses to fine-map *Vf*. M18 and AL07 were positioned at 0.2 cM and 1.1 cM from *Vf* respectively, for a total distance between the two markers of 1.3 cM. Physical mapping by pulsed-field gel electrophoresis, using M18 and AL07 as probes, demonstrated that both markers hybridize to a common 870 kb *Not*I restriction fragment. We therefore found a relationship between physical and genetic distance of 670 kb/cM in the *Vf* region. This led us to the conclusion that a chromosome walk using the recently published apple BAC library starting from M18 and AL07 is feasible.

Key words *Malus* × *domestica* · *Venturia inaequalis* · *Malus floribunda* · Disease resistance · Genetic map

Introduction

Apple scab, caused by the fungal pathogen *Venturia inaequalis* (Cke.) Wint., is the most important apple disease spread over all apple growing areas. Several sources of resistance to this disease have been described (e.g. Williams and Kuc 1969; Mac-Hardy 1996). The most-frequently used resistance gene in apple breeding programs is *Vf*, derived from *Malus floribunda* 821. *Vf* is a major dominant gene that is inherited in a Mendelian manner and influenced by minor genes that modify its activity (Gessler 1989). *Vf* is currently present in about 70 scab-resistant cultivars (Janick et al. 1996); however, the mechanism of the resistance is not yet understood.

Selection of resistant apple plants having interesting agronomic characteristics is a difficult task due to self-incompatibility and long juvenile periods. Cloning of the resistance gene will help to clarify the mechanism of resistance and could simplify the production of scab-resistant cultivars. The resistance gene alone could be introduced into well-established varieties or combined with other sources of resistance, in order to prevent or slow down resistance breakdown.

Vf has been mapped recently, and molecular markers tightly linked to the gene were found. Gessler et al. (1995) and Tartarini et al. (1999) mapped *Vf* between the molecular markers AL07 (co-segregating with AM19) and M18. M18 was positioned at a distance of 0.5 cM, while AL07 and AM19 were both mapped at 0.9 cM from *Vf* (Tartarini et al. 1999).

A method that has been shown to be successful for the isolation of genes of which only the phenotype and the map position are known is map-based cloning (Paterson and Wing 1993; Wing et al. 1994). This technique has been used for the cloning of several resistance genes such as *Pto* (Martin et al. 1993), *RPS2* (Bent et al. 1994), *Fen* (Martin et al. 1994), *Xa21* (Song et al. 1995), *Cf-2* (Dixon et al. 1996) and *Mlo* (Büschges et al. 1997), among others.

"Map-based cloning" consists of mapping the gene of interest in a large segregating population and saturating the genomic region with molecular markers to construct a fine linkage map. The physical distance separating the two closest markers bracketing the gene and the ratio between genetic and physical distance has to be determined (physical mapping). Once the maximal physical distance between the flanking markers is known, it is possible to decide whether: (1) new markers, closer to the gene, have to be found, or (2) the physical distance between the available markers could be covered using a large-insert genomic library (BAC, PAC, YAC). Once the genomic library has been selected and constructed, it is necessary to identify and align the clones in order to

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construct a contig spanning the region of interest. Finally, the resistance gene has to be identified in the selected clones, for example by phenotypic complementation in transgenic plants.

Fundamental to the success of cloning a gene by "map-based cloning" is the precise mapping of the gene and the determination that the molecular markers flanking the gene are not only genetically linked but are also physically close to the gene. It has been previously reported that markers that are genetically close have a large physical distance due to the suppression of recombination (e.g. Ganal et al. 1989; Young and Tanksley 1989). It is therefore very important to check for the absence of suppression of recombination, especially for regions introgressed from wild species, as in the case of *Vf*.

The construction of a BAC (bacterial artificial chromosome) library of the apple cultivar Florina carrying the *Vf* resistance gene has been reported recently (Vinatzer et al. 1998), so we decided to investigate the feasibility of chromosome walking towards *Vf*, starting from AL07 and M18.

In this paper we report the construction of a fine-scale genetic map around the resistance gene *Vf* and evidence that the physical distance between the two *Vf* flanking markers (AL07 and M18) does not exceed 870 kb. Moreover, we present the first results of the screening of the apple BAC library (Vinatzer et al. 1998) with the marker M18 and discuss the feasibility of cloning *Vf* using that library.

Material and methods

Plant material

The cultivar Florina was the source of DNA for the physical mapping experiments. The plants were grown in a greenhouse and young leaves were collected, immediately frozen in liquid nitrogen, and either directly used to prepare HMW-DNA or stored at −80°C.

For the fine mapping of *Vf*, young leaves from the crosses Braeburn×FAW 167 (*Vf*) and Fuji×Ariwa (*Vf*) were collected in nurseries of the FAW (Swiss Federal Research Station Wädenswil), frozen in liquid nitrogen, and freeze-dried.

Scab resistance was evaluated after greenhouse tests, as described in Gianfranceschi et al. (1996). Plants were classified for resistance into six classes according King et al. (1998).

DNA extraction for fine mapping

DNA was extracted following the protocol of Aldrich and Cullis (1993), with minor modifications. The extraction buffer was modified by adding 2% PVP40 (w/v, Polyvinylpyrrolidon) and reducing the final concentration of β-mercaptoethanol to 2% (v/v). The DNA was purified from RNA using RNaseA (Boehringer Mannheim). All the volumes were adapted to the small-scale extraction.

Fine mapping of the *Vf*-linked markers M18 and AL07

The primers of the CAPS marker M18 (Gianfranceschi et al. 1996) were modified in order to increase the annealing temperature to 60°C. The new sequences of the primers are: CH–M18for ACCACCAACTCCACCCTACA, CH–M18rev CTTTCACATGTCTAACAATTTTGG. Specific PCR amplifications of M18 and AL07 (Gessler et al. 1995) markers were performed in a volume of 15 µl containing 5 ng of genomic DNA, 1 U of Taq DNA Polymerase (Amersham-Pharmacia) 1× provided reaction buffer (50 mM KCl, 1.5 mM MgCl and 10 mM Tris-HCl), 0.1 mM dNTP (Boehringer Mannheim) and 0.2 µM of each primer. Amplifications were performed in a Perkin Elmer Cetus Gene Amp PCR System 9600. The following temperature cycles were employed: 2 min 30 s at 94°C then 35 cycles 30 s at 94°C, 30 s at 60°C and 1 min at 72°C and 1 cycle of 10 min at 72°C. Samples were kept at 4°C after amplification. M18 amplification products were then cleaved with 3 U of *TaqI* restriction enzyme (Boehringer Mannheim).

The results of the screening of the two segregating populations were added to the previously screened Florina x Nova Easygro population (491 plants, Gianfranceschi et al. 1996).

JoinMap v. 1.4 was used to construct genetic maps since this program allows the construction of a linkage map using data obtained from different segregating populations.

Preparation and embedding of intact nuclei in agarose plugs

Apple nuclei were isolated from 20 g of fresh or frozen leaves as described by Zhang et al. (1995) with the following modifications.

Anti-oxidants were added to the extraction buffer as reported by Paterson et al. (1993), and the time of the slow centrifugation (57 g) was extended to 15 min. To better-remove leaf debris, the solution containing nuclei was centrifuged a second time at 57 g and 4°C for 15 min before the last washing step. Nuclei were embedded in agarose plugs by mixing them with an equal volume (for 20 g leaves about 1 ml) of liquid 1.6% low-melting-point agarose maintained at 45°C. The mixture was then poured into ice-cold plug molds and kept on ice for 15min.

The plugs (20 mm, 10 mm, 1 mm) were subsequently incubated in the lysis buffer (0.5 M EDTA pH 9.0–9.3, 1% Sodium lauryl sarcosine, 0.1 mg/ml Proteinase K) for 36–48 h at 50°C with gentle shaking.

Digestion of the embedded HMW-DNA

After incubation in the lysis buffer the plugs were washed as described by Zhang et al. (1995). The plugs were then cut into quarters and separately equilibrated for 2 h on ice with 1 ml of 1× restriction enzyme reaction buffer and 2 mM spermidine (Fluka Chemie, Buchs, Switzerland). After 1 h the buffer was replaced, and the plugs were incubated for a second hour. After equilibration, the plugs were incubated for another hour on ice in 200 µl of 1× restriction enzyme buffer, 2 mM spermidine and 60 U of the selected restriction enzyme (Boehringer Mannheim) in order to allow the enzyme to access the DNA within the agarose plugs. Digestion was performed overnight at the recommended temperature.

The following 16 rare cutting restriction enzymes have been tested: *Cla*I, *Mlu*I, *Nae*I, *Not*I, *Sal*I, *Sfi*I, *Sma*I, *Xho*I, *Apa*I, *Bbr*PI, *Ksp*I, *Nar*I, *Pvu*I, *Spe*I, *Swa*I and *Xba*I (Boehringer Mannheim). These enzymes were chosen because they recognize either eight or or six nucleotide sequences rich in CG, which are known to be heavily methylated at the cytosine-site residue in higher plants (Van Daelen et al. 1989).

Pulsed field gel electrophoresis, blotting and hybridization

Contour-clamped homogeneous electric field (CHEF, Chu et al. 1986) electrophoresis was performed in a CHEF DR II (BioRad) system. Pulsed-field gel electrophoresis (PFGE) was performed in 1% agarose in 0.5× TBE. For the separation of the DNA fragments shown in Fig. 2, the gel was run for 22 h with an initial pulse time of 60 s and a final pulse time of 120 s at 200 V. Gels were stained with ethidium bromide (1 µg/ml) for 15 min, destained for 10 min in ddH_2O and photographed.

Table 1 Scab resistance classification of the resistant plants in the populations Braeburn×FAW 167 (*Vf*) and Fuji×Ariwa (*Vf*), and distribution of the 70 plants supposed to be wrongly classified for resistance and their relative percentage in each class

Resistance classes	0	2	3	4	Total
No. of plants per class	33	172	422	131	758
No. of plants supposed wrongly classified for resistance	4	7	27	32	70
% Plants supposed wrongly classified per class	12	4	6.4	24.4	9.2

An alkali blot of the DNA was performed on Amersham Hybond-N^+ nylon membranes using LKB 2016 vacugene vacuum blotting (Pharmacia), following the manufacturer's instructions with the following modifications: incubation time in 0.25 N HCl was extended to 40 min and blotting time was increased to 90 min.

The marker AM19 was not used as a probe because in Southern-blot experiments it hybridized to repetitive sequences. About 50 ng of the PCR-amplified M18 and AL07 fragments were purified (NuclotraPCR, labelling Macherey-Nagel) and labelled using the Pharmacia oligo-labeling kit, following the manufacturer's instructions. No further purification was performed after labelling. Overnight pre-hybridization was performed at 65°C in 30 ml of the following solution: 10% dextransulphate, 1% SDS, 50 mM Tris–HCl pH 7.5, 1 M NaCl and 600 µg of sheared and boiled salmon-sperm DNA. Hybridization was performed by adding the denaturated probe to the pre-hybridization solution and incubating at 65°C overnight. Membranes were then washed once in 2× SSC for 10 min at room temperature, once in 0.5× SSC+0.1% SDS at 65°C for 20 min and once in 0.1× SSC+0.1% SDS at 65°C for 20 min. Membranes were exposed for 2–3 days at –80°C to Kodak X-Omat AR autoradiographic films. Before re-using the membranes for hybridization they were stripped in boiling 0.1% SDS for 5 min. Probe removal was checked by exposing the membranes at –80°C on Kodak X-Omat AR autoradiographic films for 4 days.

Screening of the apple BAC library

The apple BAC library, kindly provided by H.-B. Zhang (Texas A&M University), was screened with M18 using the hybridization and washing procedure described above. The BAC plasmids were extracted by a standard alkaline miniprep (Sambrook et al. 1989).

Results

Fine mapping of *Vf*

The order of *Vf* and the markers M18 and AL07 on the genetic map has already been determined in the Florina (*Vf*)×Nova EasyGro (*Vf*) population (491 plants) (Gessler et al. 1995, 1997), generated for molecular analysis in 1994. On that population scab resistance scoring was particularly accurate, and plants with a dubious scoring have been re-checked for scab resistance in the field over several years. In this way we were able to prove that *Vf* maps between the two molecular markers, M18 and AL07.

To increase the resolution of the genetic map, the resistant plants of two other populations, Braeburn×FAW 167 (*Vf*) (279 plants) and Fuji×Ariwa (*Vf*) (409 plants), were screened with the molecular markers M18 and AL07. The plants were classified into six resistance classes according to King et al. (1998). Plants in classes 0–4 were considered to be resistant and plants in class 5 to be susceptible. Those two populations were generated for breeding purposes; therefore, their scab resistance scoring was not as careful as in the previous population. In fact, about 9% (70 out of 758) of the plants in the screening were found to be carrying both marker alleles in repulsion with *Vf*; nevertheless they were classified as resistant (Table 1). According to the order of the markers established in the Florina×Nova EasyGro population, all those plants should be considered either as double recombinants or as wrongly classified for resistance. The latter is the most probable hypothesis, and is supported by the presence of a higher percentage of misclassified plants in the classes 0 and 4. Class 0 contains plants showing no symptom of infection and thus includes those plants that "escaped" the attack of the pathogen. Class 4 includes plants showing chlorotic and necrotic spots with the presence of sporulation; however, this class is considered to be resistant. It is not always easy to distinguish class 4 from class 5, which contains the heavily infected plants with heavy sporulation that are considered to be susceptible. For the aforementioned reasons, we decided to exclude the 70 plants mentioned above for the construction of the linkage map.

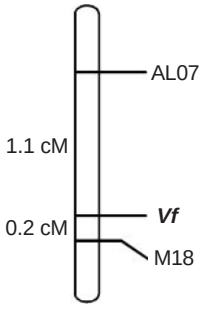

Fig. 1 Linkage map of the *Vf* genomic region. The map was calculated with the program Joinmap 1.4 using the segregation data of three populations, Florina×Nova Easygro, Fuji×Ariwa and Braeburn×FAW 167, for a total of 1179 plants analyzed

Figure 1 shows the consensus linkage map of the region obtained using data from the three populations (1179 plants). The calculated genetic distance from M18 to *Vf* and AL07 to *Vf* is 0.2 cM and 1.1 cM, respectively, covering a total of 1.3 cM.

Physical mapping

White pellets of nuclei were obtained using the modified protocol of Zhang et al. (1995). After the fixation of the nuclei into agarose plugs and subsequent incubation in lysis buffer, the plugs of some extractions turned to light brown while others remained white. This change of color is probably due to the oxidation of a little cell debris containing polyphenols, but it does not seem to influence

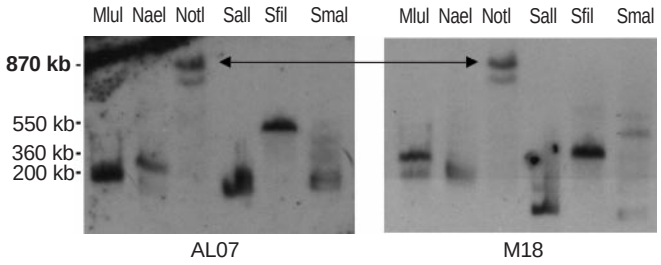

Fig. 2 Hybridization of a pulsed-field gel-electrophoresis (PFGE) gel with the two *Vf* flanking markers AL07 and M18. On the left panel the probe used was AL07, on the right one M18. The *arrow* indicates the 870 kb DNA fragment hybridizing to both markers. PFGE conditions: 1% agarose, 0.5× TBE, 14°C, 22 h with an initial pulse time of 60 s and a final pulse time of 120 s at 200 V. Yeast chromosomal DNA strain YNN295 (BioRad) was used as a size standard

Table 2 Size of PFGE-separated restriction fragments (in kb) hybridizing to the markers AL07 and M18

Fragment	Probe	
	AL07	**M18**
*Mlu*I	200 kb	300 kb
*Nae*I	230 kb	170 kb
*Not*I	870 kb	870 kb
*Sfi*I	550 kb	360 kb
*Sma*I	150 kb	400 kb
*Apa*I, *Cla*I, *Ksp*I and *Pvu*I	>1,600 kb	>1,600 kb
*BbrP*I, *Nar*I, *Sal*I, *Spe*I, *Swa*I, *Xba*I, and *Xho*I	<100 kb	<100 kb

the digestion of the DNA. Pulsed-field gel electrophoresis (PFGE) of undigested and digested HMW-DNA proved that the extracted DNA is of megabase size and accessible to restriction enzymes (data not shown).

Out of the 16 rare-cutting restriction enzymes tested, only *Not*I produced a fragment that hybridized to both *Vf* flanking markers, AL07 and M18 (Fig. 2). The size of this fragment is about 870 kb. Since the genetic distance between AL07 and M18 is 1.3 cM (Fig. 1), we could calculate the maximum specific ratio between physical and genetic distance for the *Vf* region to be 670 kb/cM.

Other restriction enzymes, such as *Mlu*I, *Sfi*I, *Sma*I and *Nae*I, produced fragments hybridizing to either marker ranging from 200 kb to 550 kb, but no other restriction fragment on which both markers could hybridize was found. Restriction enzymes *Apa*I, *Ksp*I, *Pvu*I and *Cla*I did not produce fragments in the resolution range of the chosen PFGE settings. The HMW-DNA fragments of those restriction enzymes hybridizing to the probes were in the compression band, indicating that their size is bigger than 1.6 Mbp. All other restriction enzymes tested produced fragments hybridizing to the probes smaller than 100 kb (Table 2).

In Fig. 2, in the lanes of the restriction enzymes *Mlu*I and *Not*I, two bands appear. This can be explained by a restriction polymorphism (Florina is heterozygous for *Vf*) which could be caused not only by point mutations modifying the recognition site but also by a different methylation of the site.

Screening of the apple BAC library with M18

Screening of the BAC library with the M18 marker led to the identification of three positive clones. Two of these clones contain the allele in repulsion with *Vf*, and one carries the allele in coupling with *Vf*. Surprisingly, all three BAC inserts have a *Not*I restriction site. This indicates that the maximal distance between M18 and the *Not*I restriction site is 80 kb, which is the size of the smaller BAC insert.

Discussion

The haploid genome of *Malus* × *domestica* is estimated to be between 743–796 Mb (Arumuganathan and Earle 1991). The published linkage maps of apple range from 692 cM (Conner et al. 1997) to 984 cM (Maliepaard et al. 1998); by assuming an average haploid genome size of 770 Mbp, 1 cM would correspond, for the largest linkage map, to about 780 kb/cM, and for the smallest to 1110 kb/cM.

The results – a maximal physical distance of 870 kb and a genetic distance of 1.3 cM between the two *Vf* flanking markers – lead to a specific ratio between genetic and physical distance for the *Vf* region of 670 kb/cM. This value is not very different from the estimated value obtained with the largest linkage map of Maliepaard (780 kb/cM), indicating the absence of suppression of recombination in the region. In fact, where suppression of recombination was found, as in the Tm-2a region, the two values diverged by a factor of seven (Ganal et al. 1989).

The fact that recombination suppression was not observed could indicate that the introgressed region from the wild apple species is not substantially different from the one present in *Malus* × *domestica*. However, the possibility that suppression of recombination could be found in a smaller region between the two markers AL07 and M18 should not be excluded.

Our results indicate that a chromosome-walking project using BAC clones is feasible; in fact, according to our estimations M18 should be only 130 kb away from *Vf*. However, it is difficult to determine the precise location of *Vf* on the linkage map, since phenotypic data are not as precise as molecular data. In fact, during the analysis of the populations Braeburn×FAW 167 (*Vf*) and Fuji×Ariwa (*Vf*), we found about 9% of plants classified as resistant but showing the alleles of both markers in repulsion with *Vf*. To explain this result two hypotheses can be formulated: either the plants are double recombinants, or else they were wrongly classified for resistance.

The first hypothesis is quite unlikely, because given that the genetic distance between M18 and *Vf* and AL07

and *Vf* are 0.5 and 0.9 cM respectively, as reported by Tartarini et al. (1999), the probability of double crossing-over events cutting out the resistance gene should be 9×10^{-5}. The value of 9% observed in our populations is significantly different from the expected one, being 1000-times bigger.

The second hypothesis, a wrong classification of the resistance, is the most probable, and is supported also by the fact that most of the plants showing the supposed double crossing-over belong to resistance classes 0 and 4, classes known to be critical for the scoring of resistance. An indirect validation of this hypothesis comes also from the fact that if the scoring of *Vf* in these plants were to be considered correct, the markers AL07 and M18 should map very far from *Vf*; this would be in disagreement with our Florina×Nova Easygro data and also with works previously published by Gardiner et al. (1996); Hemmat et al. (1998); Tartarini et al. (1996); King et al. (1998), and Maliepaard et al. (1998).

The construction of a BAC library with inserts of the cultivar Florina (*Vf*) has been reported recently (Vinatzer et al. 1998). The average size of the inserts is about 120 kb. That means that with an average "walking-step" of 60 kb, the *Vf* region between the two markers should be completely covered in 12–14 steps.

We started the screening of such a BAC library with the marker M18 and found three positive clones whose inserts were cleaved by *Not*I. The screening of the same apple library with AL07 gave six positive clones; two of them, one containing the allele of AL07 in coupling with *Vf* and the other the one in repulsion, were also cleaved by *NotI* (Vinatzer, personal communication). These results indicate that the distance between AL07 and M18 should not be very different from 870 kb (870 kb is the maximal distance in case the two markers are very near to the two *Not*I restriction sites).

To further confirm the estimation of the physical distance during the chromosome walking, we will determine if the clones of the contig contain *Not*I restriction sites. However, the final confirmation of the physical distance between the two markers will be obtained only when the contig spanning the genomic region is generated.

Thanks to the large number of plants analyzed, it should be relatively easy to determine the direction of the walk in the first steps of chromosome walking and afterwards to reduce to the minimum the genomic region where the *Vf* gene should be located.

In conclusion, the results of the physical mapping and the availability of large segregating populations indicate that a chromosome-walking project starting from the markers M18 and AL07 using the available BAC library is feasible. The number of steps necessary to find all clones covering the region of interest should not exceed 12–14.

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