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Saturation mapping of the apple scab resistance gene *Vf* using AFLP markers

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Abstract Using the amplified fragment length polymorphism (AFLP) technique combined with a "narrow-down" bulk segregant strategy enabled us to quickly identify 15 tightly linked AFLP markers to the *Vf* gene that confers resistance to the apple scab disease. High-resolution mapping placed all 15 AFLP markers within an interval of 0.6 cM around the *Vf* region; 7 of them were found to be inseparable from the *Vf* gene, 1 was localized left of, and the remaining 7 located right of the *Vf* gene. In addition, eight previously identified RAPD markers were also mapped, but only three, including M18, AM19, and AL07, were localized within this short interval, and none co-segregated with the *Vf* gene. The saturation of the *Vf* region with AFLP markers will accelerate both marker-assisted selection and map-based cloning. The advantages of this "narrow-down" strategy, estimation of physical distances among AFLP markers, and their potential application are also discussed.

Key words Apple · Scab resistance · Mapping · *Vf* gene · AFLP marker

Introduction

Apple scab, incited by the fungal pathogen *Venturia inaequalis* (Cke.) Wint., is one of the most serious diseases of apple. The disease weakens trees and damages fruit, hence shortening the life of the orchard and rendering fruit unsaleable on the fresh market. Almost all apple cultivars grown commercially around the world are susceptible to this disease. Chemical control of apple scab in orchards requires 12–15 sprayings of fungicides annually, thus increasing production cost, causing environmental pollution, and probably affecting the consumer's health. As an alternative, a genetic approach by incorporating a scab resistance gene into commercial apple cultivars has so far been the most promising option to control the disease.

At least six major independent genes conferring resistance to *V. inaequalis* have been identified in apple germplasm: *Vf* from *Malus floribunda* 821, *Vm* from *M. micromalus*, *Vb* from *M. baccata* Hansen's no. 2, *Vbj* from *M. baccata* var. *jackii*, *Vr* from *M. pumila* R12740–7A, and *Va* from the Antonovaka PI 1726623 (Williams and Kuc 1969; Biggs 1990). To date, only the *Vf* gene has been widely introgressed into susceptible commercial apple cultivars (Granger et al. 1997; Korban 1998). Of the 52 scab-resistant cultivars named and released, over 75% carry the *Vf* gene (Korban and Chen 1992). The *Vf* gene confers resistance to all five known races of *V. inaequalis* and has held up very well in orchards for over 80 years. Recently, a new race of *V. inaequalis* (designated race 6), first identified in Germany and later in other regions of Europe, has been found to be able overcome the *Vf* gene in some genetic backgrounds (Parisi et al. 1993; Parisi and Lespinasse 1996). Therefore, pyramiding of the *Vf* with other *V* genes is likely to achieve a more durable resistance.

The identification of molecular markers linked to the *Vf* gene is particularly useful in both marker-assisted selection (MAS) and map-based gene cloning (King et al. 1999; Tanksley et al. 1995) and thus has attracted the attention of many researchers around the world. So far, an isozyme marker, *Pgm-1* (Korban and Bournival 1987; Manganaris et al. 1994), and a number of restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) markers have been found to be linked to the *Vf* gene (Yang and Korban 1996; Tartarini 1996; Gardiner et al. 1996; Gianfranceschi et al. 1996; Yang et al. 1997 a, b; Hemmat et al. 1998). Furthermore, nearly all RAPD markers have been converted into reliable polymerase chain reaction (PCR)-based markers, namely sequence-

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characterized amplified regions (SCARs) (Yang and Korban 1996; Yang et al. 1997a, b; Hemmat et al. 1998; Tartarini et al. 1999) or cleaved-amplified polymorphic sequences (CAPs) (Gianfranceschi et al. 1996). However, because of the low efficiency of the RAPD method in searching for linked markers, only eight markers have been so far mapped within 10 cM of the *Vf* gene. It has been difficult to further saturate the *Vf* region with RAPD markers. Hence, a more powerful marker technique is needed to identify more tightly linked markers for both MAS and *Vf* gene cloning.

Based on known RFLP, RAPD, and isozyme markers, several linkage maps around the *Vf* region have been published (Gardiner et al. 1996; King et al. 1998; Hemmat et al. 1998). Unfortunately, the order and distance of the existing markers are not compatible among the different linkage maps. For example, Hemmat et al. (1998) mapped the markers M18 and AL07 on the same side of the *Vf* gene, while King et al. (1998) placed these two markers on opposite sides of the *Vf* gene. Considering that these two markers are the closest found so far to the *Vf* gene, and have already served as starting points in map-based cloning of the *Vf* gene (Patocchi et al. 1999a,b), it is critical that they are correctly placed along the map of the *Vf* region.

The amplified fragment length polymorphism (AFLP) technique combined with bulk segregant analysis is at present considered to be the most powerful method in both searching for linked markers and for saturating the target region (Vos et al. 1995; Ballvora et al. 1995; Thomas et al. 1995; Qi et al. 1998; Xu et al. 1999). Normally, 50–100 AFLP loci can be surveyed with every primer combination. By increasing the number of primer combinations, large numbers of AFLP loci can be screened throughout the whole genome, thus enabling AFLP markers tightly linked to the target gene to be found. Thomas et al. (1995) obtained two markers located on opposite sides of the *Cf* gene in tomato separated by only 15.5 kb of intervening DNA by screening about 42,000 AFLP loci.

The objective of the research presented here was to saturate the *Vf* region with AFLP markers and construct a high-resolution map around the *Vf* gene.

Materials and methods

Plant material

The following genetic materials were used to identify closely linked AFLP markers: (1) *M. floribunda* clone 821, the original source of the *Vf* scab resistance gene; (2) scab-resistant apple selections (Co-op selections) developed from the breeding programs of the University of Illinois in collaboration with Purdue and Rutgers universities (PRI program); and (3) ten susceptible apple cultivars including Delicious, Empire, Gala, Golden Delicious, Idared, Jonathan, McIntosh, Red Delicious, Rome Beauty, and Yellow Newton. The following three populations were used for high-resolution mapping: (1) 38 resistant Co-op selections (Co-op 1 to Co-op 38), (2) 203 F_1 resistant individuals from the cross Co-op 17×Co-op 16, and (3) 227 F_1 resistant individuals from the cross Jonafree×Ill. Del. no. 1.

DNA extraction

Apple genomic DNA was extracted from fresh leaf tissue using the Nucleon HytoPure plant DNA extraction Kit (Amersham Life Science). Approximately 0.1 g of leaf tissue was frozen in liquid nitrogen, pounded to pieces with a bamboo skewer, and ground to a fine powder with steel beads on a high-speed vortex. The DNA extraction followed the procedure described by the supplier.

"Narrow-down" bulk segregant analysis to identify AFLP markers linked to the *Vf* gene

The Co-op selections were developed from the PRI breeding program by introducing the *Vf* gene from *M. floribunda* clone 821 into susceptible commercial apple cultivars, and they are at least five to six generations removed from *M. floribunda* clone 821. Consequently, all Co-ops are characterized by sharing the same *Vf* gene, containing different sizes of the introgressed region, and having a low background from the *M. floribunda* 821 genome. Therefore, these Co-ops are of particular interest in searching for linked markers to the *Vf* gene using the bulk segregant strategy of Michelmore et al. (1991) and as modified by Yang et al. (1997b).

As a first step of the "narrow-down" bulk segregant strategy, equal amounts of DNA from Co-ops 1 to 38 were pooled to form a resistant bulk (Rb), while genomic DNA from the ten commercial apple cultivars were pooled to produce the contrasting susceptible bulk (Sb). These two bulks, together with *M. floribunda* 821, were used to screen AFLP markers linked to the *Vf* gene. AFLP bands that were present in both *M. floribunda* 821 and the resistant bulk but absent in the susceptible bulk were most likely derived from the *Vf* region. All putative linked AFLP markers were then used to check all Co-op selections individually and to construct a rough map around the *Vf* gene. Meanwhile, the extent of the introgressed region encompassing the *Vf* gene could be roughly inferred for each Co-op selection according to the mapping results. In the second step of the "narrow-down" strategy, the resistant bulk was split into two sub-bulks. One sub-bulk, designated Rb1, was composed of those Co-op selections having the short introgressed region to the left of the *Vf* gene. The other sub-bulk, designated Rb2, consisted of those Co-op selections having the short introgressed section to the right of the *Vf* gene. AFLP bands that were simultaneously present in *M. floribunda* 821, Rb1, and Rb2, but not in Sb, were assumed to be derived from the short introgressed region common to both Rb1 and Rb2. These putative linked AFLP markers were in turn used to map the *Vf* gene to estimate the extent of the introgressed region present in each Co-op selection. The more closely the AFLP markers obtained were linked, the more detailed the physical map of the introgressed region for each Co-op selection. Finally, the number of Co-op selections that made up each of the two sub-bulks, Rb1 and Rb2, could be reduced down to one single individual, thus narrowing down the screened region to a bare minimum. AFLP bands derived from such a short region were then undoubtedly the most closely linked to the *Vf* gene.

AFLP analysis

AFLP analyses were performed according to the supplier's instructions (GibcoBRL, Life Technologies) and as described in detail by Vos et al. (1995). A total of 1,024 primer combinations derived from 32 *Eco*RI primers and 32 *Mse*I primers were employed in this study. Those 32 *Eco*RI primers included 16 E-Axx (x represents one of the four bases A, T, G, and C) and 16 E-Txx. Likewise, the 32 *Mse*I primers included 16 M-Cxx and 16 M-Gxx. The primer combination was represented as EA(orT)*m*MC(G)*n* (m and n corresponding to numbers from 1 to 16). A linked AFLP marker was designated as *EA*(or*T*)*mMC*(*G*)*n-1* for the first marker, and *EA*(or*T*)*mMC*(*G*)*n–2* for the second marker, and so on.

High-resolution mapping

In the mapping experiment, closely linked AFLP markers identified above along with eight previously reported linked markers

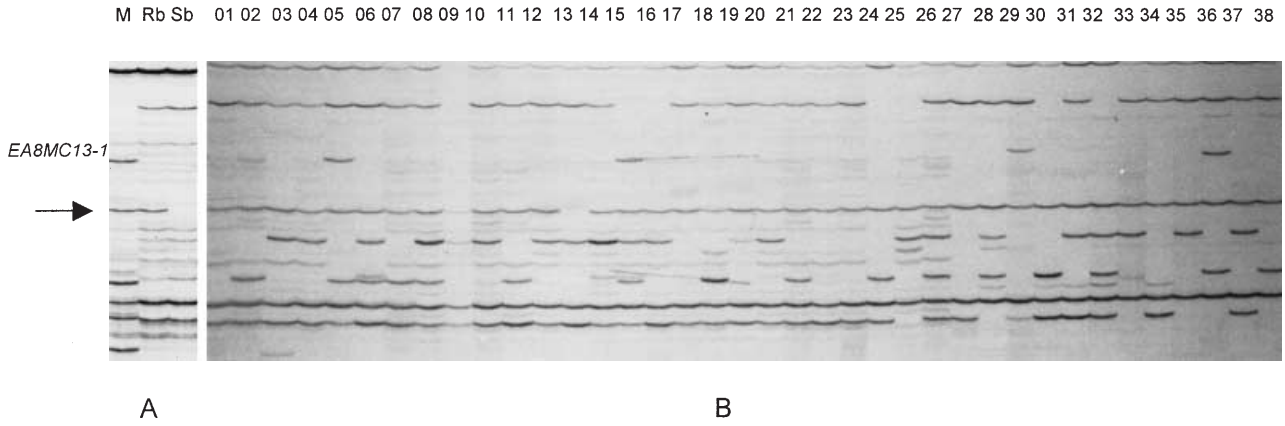

Fig. 1A, B Screening of putative AFLP markers using three DNA pools. **Panel A** (on the *left*) A putative linked AFLP marker *EA8MC13–1* was present in both *M. Floribunda 821* (*M*) and the resistant bulk (*Rb*), but not in the susceptible bulk (Sb). **Panel B** (on the *right*) Thirty-eight Co-op selections were used to map the putative linked AFLP marker *EA8 MC13–1*. As shown in the figure (arrow), the linked AFLP marker *EA8MC13–1* was present in all Co-op selections except for Co-op 13. *Lane numbers 1–38* correspond to Co-op selections 1–38

were included. The latter group included seven SCARs, $OPD20_{600}$, $OPK16_{1300}$, $OPAR4_{1400}$ (Yang and Korban 1996; Yang et al. 1997 a,b), $S5_{1450}$, $U1_{400}$ (Hemmat et al. 1998), AL07, and AM19 (Tartarini et al. 1999), and one CAP, M18 (Gianfranceschi et al. 1996). All individuals from the three mapping populations described above were tested for their genotypes using these seven SCARs, one CAP, and all closely-linked AFLP markers. A joint high-resolution map for the *Vf* region was constructed using CRI-MAP software (Green et al. 1990). Map distances were calculated by using the mapping function of Kosambi (1944).

Results

AFLP profile for *M. floribunda* 821 and apple cultivars

On average, each AFLP primer combination produced 74 bands for *M. floribunda* 821 and 78 bands for the susceptible apple bulk. Comparing AFLP banding patterns, we found only 44 AFLP bands to be common between these two DNA samples. The remaining 30 AFLP bands in *M. floribunda* 821 and 34 in the susceptible bulk were found to be polymorphic. The high level of polymorphism demonstrated the large difference in the genomic sequence between the cultivated apple, *M.×domestica*, and the wild species, *M. floribunda* 821. As our objective was to identify AFLP markers linked in *cis* with the *Vf* gene, only polymorphic bands from the *M. floribunda* 821 were deemed informative. With 1,024 primer combinations, approximately 30,000 informative AFLP bands were scored. If these informative bands were distributed equally, and the size of the *M. floribunda* 821 genome was 10^6 kb, as estimated by Chen (1989), then this represented an average of 1 AFLP marker every 20 kb.

Screening of putative AFLP markers with three DNA pools

In the first step of the "narrow-down" strategy, 128 primer pairs derived from combining 8 *Eco*RI primers (EA1 to EA8) with 16 *Mse*I primers (MC1 to MC16) were used to investigate the following three DNA samples: *M. floribunda* 821, resistant bulk (Rb), and susceptible bulk (Sb). In total, 32 AFLP bands were found to be present in both *M. floribunda* 821 and Rb but not in Sb (Fig. 1A). After mapping these markers in individuals in the Co-op material (see Materials and methods), only 8 AFLP bands were found to be linked to the *Vf* gene. One of these AFLP markers, *EA8MC13–1*, appeared to be tightly linked to the *Vf* gene, as it was present in 37 out of 38 Co-op selections (Fig. 1B). Meanwhile, the extent of the introgressed region for each Co-op selection was roughly estimated using these 8 AFLP markers. Seven Co-ops (Co-ops 13, 15, 16, 29, 30, 34, and 36) contained relatively short introgressed sections left of the *Vf* gene. DNA from these 7 Co-ops were pooled together to form Rb1. Another 5 Co-ops (Co-ops 5, 7, 8, 11, and 27) contained relatively short introgressed sections right of the *Vf* gene. Likewise, DNA from these 5 Co-ops were pooled together to form Rb2.

Screening of tightly linked AFLP markers with four DNA samples

A total of 128 primer combinations of yet another 8 *Eco*RI primers (EA9 to EA16) and 16 *Mse*I primers (MC1 to MC16) were used to screen the four DNA samples described above (*M. floribunda* 821, Rb1, Rb2, and Sb). Consequently, 31 additional putative linked AFLP markers were observed, which appeared in *M. floribunda* 821, Rb1, and Rb2, but not in Sb (Fig. 2A). A rough mapping of these putative linked AFLP markers was carried out using all 38 Co-op selections individually. A total of 11 out of these 31 AFLP bands were mapped within the *Vf* region. One AFLP marker, *EA9MC15–1*, was present in all 38 Co-op selections and thus was inseparable from the *Vf* gene within this Co-op material. Three AFLP markers, *EA11MC7–1*, *EA16MC13–1*, *and EA12MC5–1*, showed a band in all Co-ops, with the exeption of Co-op 13 and thus

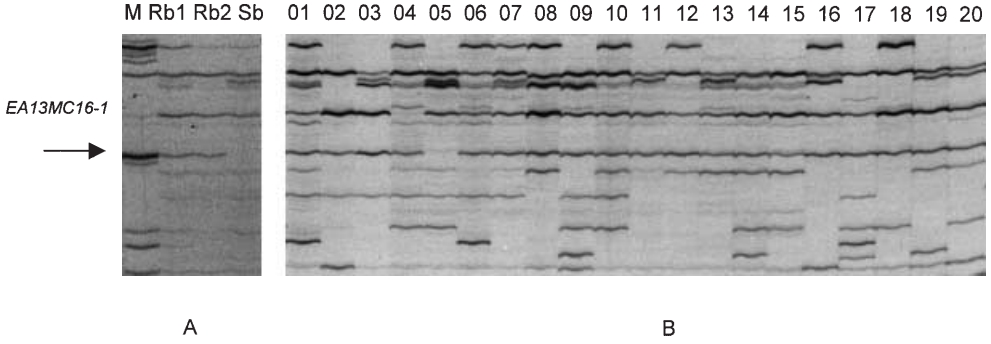

Fig. 2A, B Screening of tightly linked AFLP markers using four DNA pools. **Panel A** (on the *left*) A putative linked AFLP marker *EA13MC16–1* (*arrow*) was present in *M*, *Rb1*, and *Rb2*, but not in *Sb*. *M M. floribunda 821*, *Rb1* resistant bulk 1, consisting of 7 Co-op selections (Co-ops 13, 15, 16, 29, 30, 34, and 36), *Rb2* resistant bulk 2, consisting of 5 Co-op selections (Co-ops 5, 7, 8, 11, and 27). **Panel B** (on the *right*) The putative linked AFLP marker *EA13MC16–1* (*arrow*) was mapped using Co-op selections. It was present in all Co-op selections but Co-op 8 (*lane 5*). *Lane numbers 1–20* correspond to Co-ops 4, 5, 6, 7, 8, 10, 11, 13, 14, 15, 16, 17, 18, 20, 25, 27, 29, 33, 34, and 36, respectively

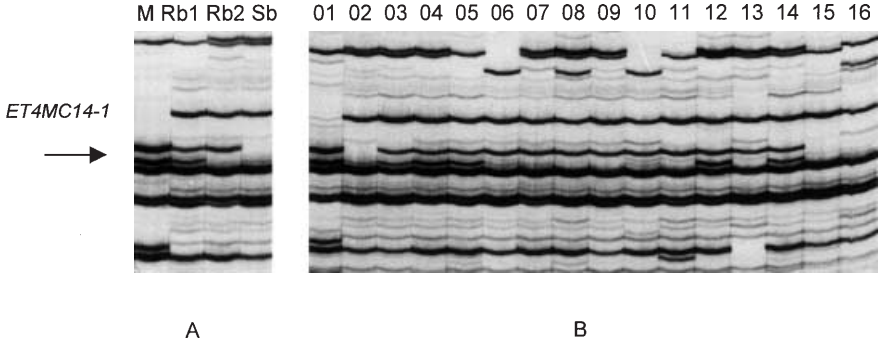

Fig. 3A, B Screening of tightly linked AFLP markers using four DNA pools. **Panel A** (on the *left*) A putative linked AFLP marker *ET4MC14–1* (*arrow*) was present in *M*, *Rb1*, and *Rb2*, but not in *Sb*. *M M. floribunda 821*; *Rb1* resistant bulk 1, consisting of only single Co-op 13, *Rb2* resistant bulk 2, consisting of only single Co-op 8, *Sb* susceptible bulk. **Panel B** (on the *right*). The putative linked AFLP marker *ET4MC14–1* (*arrow*) was mapped using Co-op selections. It was present in all Co-op selections. *Lane numbers 1–16* correspond to *M. floribunda 821*, Sb, Co-ops 5, 7, 8, 11, 13, 15, 16, 27, 29, 30, 34, 36, and susceptible apple cultivars Golden Delicious and Rome Beauty, respectively

Table 1 Presence/absence of 18 closely linked markers in 5 selected individuals from three scab-resistant populations[a]

	Selected individuals from these resistant populations[a]				
	P9–1–202	P9–2–17	P9–2–38	P9–2–59	P2–3–117
ET9MC3–1[b]	0	1	0	0	0
EA2MG11-1[c]	1	1	0	1	0
ET9MC15-1[d]	1	0	0	1	0

[a] A total of 470 individuals from three resistant populations have been screened with all 18 closely linked markers. Presence/absence of markers in 5 individuals are listed in the table. These 5 individuals were selected as having the shortest donor region left of (P9–1-202, P9–2-59) and right of (P9–2-17) the *Vf* gene, or without a donor region (P9–2-38, P2–3-117)

[b] A similar segregation pattern was also observed for M18, a CAP marker left of the *Vf* gene identified by Gianfranceschi et al. (1996)

[c] A similar segregation pattern was also observed for *EA12MG16–1, EA11MG4–1, ET2MC8–1, ET3MG10–1, ET8MG1–1*, and *ET8MG7–1*, all AFLP markers identified in this study

[d] A similar segregation pattern was also observed for *EA4MG1–1, EA16MG2–1, ET4MC14–1, ET3MG10–2, ET8MG16–1, ET10MG8*-1 (all AFLP markers identified in this study), and for AM19 and AL107, both SCAR markers found to the right of the *Vf* gene, identified by Tartarini et al. (1999)

were mapped left of the *Vf* gene. Another 3 AFLP markers, *EA13MC16–1, EA14MC1–1, and EA16MC3–1*, were present in all Co-ops except for Co-op 8 and thus were placed right of the *Vf* gene (Fig. 2B). The remaining 4 AFLP markers were not so closely linked to the *Vf* gene due to their absence in many of the Co-op selections. The mapping results also revealed that Co-op 13 had the shortest introgressed section left of the *Vf* gene, while Co-op 8 had the shortest introgressed section right of the *Vf* gene. Therefore, these 2 Co-ops were ideal for screening this

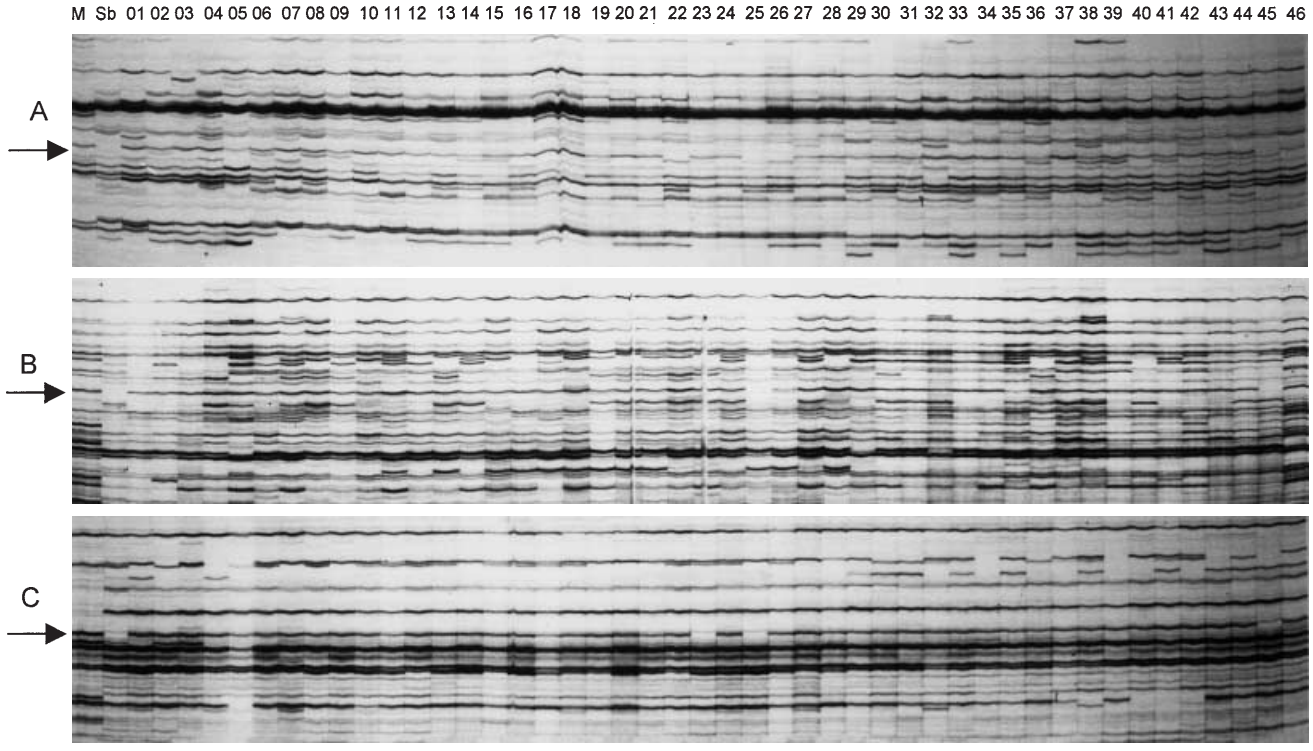

Fig. 4A–C Joint-mapping of the *Vf* gene using three scab-resistant populations (all Co-op materials; seedlings from cross Co-op17×Co-op 16; seedlings from Jonafree × Ill. Del. no. 1). A total of 46 individuals from the three resistant populations was used in the final fine-mapping of closely linked AFLP markers identified in this study as well as those previously identified linked RAPD markers. These individuals were selected due to their shorter introgressed region left or right to the *Vf* gene. None of the linked markers tested in this study were present in 2 individuals, P9–2-38 (*lane no. 25*) and P2–3-117 (*lane no. 45*), thus they were deemed as escapes. **Panel A** (at the *top*) Fine-mapping of the linked AFLP marker *ET9MC3–1* (*arrow*), which was present in all individuals except for the 2 escape individuals (P9–2-38 and P2–3-117) and another 2 individuals P9–1-202 (*lane no. 22*) and P9–2-59 (*lane no. 26*), and thus was mapped left to the *Vf* gene. **Panel B** (in the *middle*) Fine-mapping of the linked AFLP marker *ET8MG7–1* (*arrow*), which was present in all individuals but 2 escape individuals (P9–2-38 and P2–3-117), and thus co-segregated with the *Vf* gene. **Panel C** (at the *bottom*) Fine-mapping of the linked AFLP marker *ET4MC4–1* (*arrow*), which was present in all individuals except for the 2 escape individuals (P9–2-38 and P2–3-117) and 1 other individual P9–2-17 (*lane no. 23*), and thus was mapped to the right of the *Vf* gene. *M M. floribunda 821*; *Sb* susceptible bulk

material for very closely linked AFLP markers. Any AFLP markers derived from the narrow introgressed section common to both Co-op 13 and Co-op 8 would be closest to the *Vf* gene.

An additional 768 primer pairs derived from combining 16EAxx and 16 MGxx, 16 ETxx and 16 MCxx, and 16 ETxx and 16 MGxx were used to screen a new set of four DNA pools (*M. floribunda* 821, Co-op 13, Co-op 8, and Sb). Any putative closely linked AFLP markers would appear in *M. floribunda* 821, Co-op 13, Co-op 8 but not in Sb (Fig. 3A). As a result, 94 such AFLP bands were found. Likewise, mapping of these AFLP markers to the *Vf* gene was conducted using the Co-op material. Only 14 AFLP bands (*EA2MG11–1*, *EA4MG1–1*, *EA11MG4–1*, *EA12MG16–1*, *EA16MG2–1*, *ET2MC8–1*, *ET4MC14–1*, *ET9MC3–1*, *ET3MG10–1*, *ET3MG10–2*, *ET8MG1–1*, *ET8MG7–1*, *ET8MG16–1*, *ET10MG8–1*) were identified to be linked markers. Each of these was so very closely linked to the *Vf* gene that no recombinant was found between these markers and the *Vf* gene within this Co-op material (Fig. 3B).

Joint mapping of the *Vf* gene using three scab-resistant populations

Eight previously found markers around the *Vf* region were also included for high-resolution mapping in the present study. These were initially mapped using the Co-op material. It was found that M18, AM19, and AL07 were inseparable from the *Vf* gene, while OPK16, OPAR4, and UI400 were located on the left side of the *Vf* gene. OPD20 and S5 were placed on the right side of the *Vf* gene. The order of these eight markers was similar to that reported by others (King et al. 1998; Hemmat et al. 1998). Because of the limited number of individuals of the Co-op material, it was impossible to resolve the three closest markers, M18, AM19, and AL07.

Two seedling populations having a total of 430 resistant individuals were employed for high-resolution mapping in our study. The markers selected for mapping included eight previously reported linked markers (as described above), 15 co-segregating AFLP markers, 2 AFLP markers (*EA8MC13–1* and *EA5MG3–1*) left of, and 1 AFLP marker (*EA13MC16–1*) right of the *Vf* gene (all AFLP markers were found in this study). Mapping

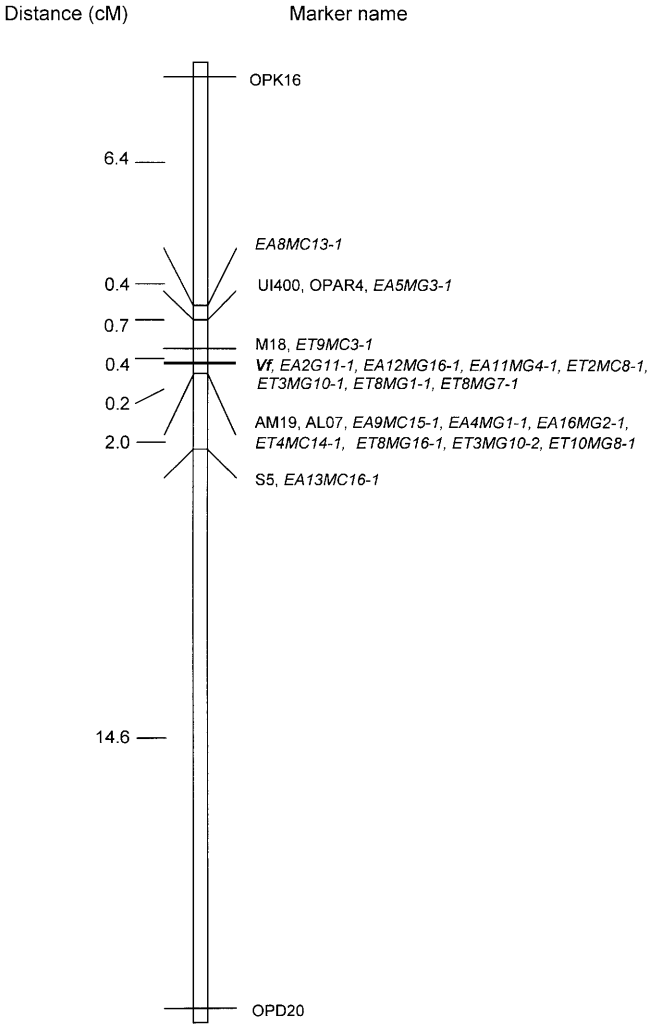

Fig. 5 A high-resolution linkage map of the *Vf* gene based on three resistant populations

these markers using the Co-op material enabled us to roughly detect the order and genetic distances of all these markers. In order to map all linked markers efficiently using two large seedling resistant populations, we initially used the two remote markers OPK16 and OPD20 located on opposite sides of the *Vf* gene to check all 430 resistant individuals. Only recombinants identified in both OPK16/*Vf* and OPD20/*Vf* intervals were further used in the mapping of all other markers located within the OPK16/OPD20 interval.

Out of 430 resistant seedling individuals, 2 individuals, P9–2-38 and P2–3-117, were deemed escapes, as none of the tested markers were present in these 2 individuals (Table 1; Fig. 4A–C), and they were thus eliminated from the mapping analysis. For those eight previously identified markers, M18 was the closest left of the *Vf* gene, as only 2 recombinants (P9–1-202 and P9–2-59) were found within the M18/*Vf* interval. On the right side of the *Vf* gene, AM19 and AL07 were the closest markers, and both located at the same position with only 1 recombinant (P9–2-17) found between them and the *Vf* gene (Table 1). OPAR4 and UI400 were located beyond M18, and S5 was placed beyond AM19. As to the AFLP markers, 7 (*EA2MG11–1*, *EA11MG4–1*, *EA12MG16–1*, *ET2MC8–1*, *ET3MG10–1*, *ET8MG1–1*, and *ET8MG7–1*) were so close to the *Vf* gene that no recombinant was identified in the two resistant seedling populations. One marker (*ET9MC3–1*) produced 2 recombinants (P9–1-202 and P9–2-59), similar to M18, and hence was placed at the same position as M18. As many as 7 AFLP markers (*EA9MC15–1*, *EA4MG1–1*, *EA16MG2–1*, *ET4MC14–1*, *ET3MG10–2*, *ET8MG16–1*, and *ET10MG8–1*) revealed 1 recombinant (P9–2-17), similar to AM19 and AL07, and therefore all these markers were clustered at the same position (Table 1, Fig. 4A–C).

A joint map of the two resistant seedling populations as well as the Co-op material was constructed for all markers using the CRI-MAP mapping software. The order and the genetic distance between markers are shown in Fig. 5. Seven AFLP markers are located at the same position as the *Vf* gene; M18 and *ET9MC3–1* are 0.4 cM left of the *Vf* gene; AM19/AL07 together with 7 other AFLP markers are 0.2 cM right of the *Vf* gene.

Discussion

In the present study, the AFLP technique has proven to be a powerful tool for identifying tightly linked markers which are indispensable to marker-assisted selection and to proceeding with map-based gene cloning. Using 1,024 AFLP primer pairs, we obtained 15 markers within 0.6 cM of the *Vf* region, 7 of which are inseparable from the *Vf* gene based on three mapping populations of approximately 470 resistant individuals. In previous studies in which the RAPD technique was used, only three markers (M18, AM19, and AL07) were identified within this narrow region (Tartarini et al. 1999; Gianfranceschi et al. 1996), none of which co-segarated with the *Vf* gene. Patocchi et al. (1999b) have reported the physical distance separated by M18 and AM9/AL07 to be approximately 550 kb. If we are to assume that the 15 identified AFLP markers are equally distributed within this region, this will result in an average of 1 AFLP marker in about 40 kb. Such a high-density marker ratio within the *Vf* region enables us to adopt a positional cloning strategy to clone this gene from an apple BAC library. For future use of these AFLP markers for screening an apple BAC library, all AFLP markers will be converted into PCR-based markers.

Theoretically, the physical distance between AFLP markers depends only on two factors – the level of polymorphism between two parents and the number of primer pairs used. In our AFLP analysis, a less frequent cutter enzyme, *Eco*RI, and a frequent cutter enzyme, *Mse*I, are used together to digest genomic DNA. *Eco*RI has a six-base recognition site and on average represents one recognition site in 4,096 bp; *Mse*I has a four-base recognition site and on average represents one site in 256 bp. There are three types of fragments (*Eco*RI-*Eco*RI; *Mse*I-*Mse*I; and *Eco*RI-*Mse*I) generated following double-di-

gestion with *Eco*RI and *Mse*I. Due to primer design and the amplification strategy, only the *Eco*RI-*Mse*I fragments are preferentially amplified and scored in an AFLP analysis. The number of *Eco*RI-*Mse*I fragments is approximately twice that of *Eco*RI recognition sites. That is to say, 1 *Eco*RI-*Mse*I fragment can be generated once every 2 kb. Usually, not all *Eco*RI-*Mse*I fragments are amplified. The ratio of amplified *Eco*RI-*Mse*I fragments to total number of *Eco*RI-*Mse*I fragments depends on the number of primer combinations used. As for those amplified *Eco*RI-*Mse*I fragments (or AFLP bands), only polymorphic bands are informative and can be used to search for AFLP markers linked to the gene of interest. Taking these factors into account, we propose the following formula to estimate the average physical distance (PD) between linked AFLP markers: PD (kb)=2.048× 4,096/r×y. In this formula, the numerical factor 2.048 (kb) corresponds to the average distance between any 2 neighboring *Eco*RI-*Mse*I fragments; the number 4,096 corresponds to the total number of combinations of AFLP primers having three selective base pairs; r represents the ratio of polymorphic bands to total number of bands in the original source of the target gene (i.e. *M. floribunda* 821); y is the number of primer pairs used. In the present study, r is 0.4, y is 1,024, and therefore the physical distance between two AFLP markers is calculated to be 20 kb. This distance is the same as the estimated value reported earlier in our AFLP analysis (see Results). Also, this formula can confirm our estimation based on the findings of Patocchi et al. (1999a) that the average distance of an AFLP marker within the *Vf* region is about 40 kb. Given that the ratio of polymorphism is over 10% between the two parents, 1,024 primer pairs will generate linked AFLP markers having an average distance of 80 kb. Such closely-linked AFLP markers are useful for undertaking a positional cloning strategy. The size of the genome only has a relationship to the number of AFLP bands observed with each primer pair used. The larger the genome size, the more bands that will appear for each primer pair. Normally, with three selective base pairs for both the *Eco*RI and the *Mse*I primer and a genome size of 1×10^6 kb, the number of AFLP bands will range between 50 and 100. Polyacrymide or Long-Ranger sequence gels are capable of resolving 50–100 AFLP bands.

The "narrow-down" strategy adopted in this study has been found to be useful in efficiently identifying closely linked markers. The rough-mapping results obtained in the first step of this strategy indicate that most Co-op selections contain large introgressed segments, although all these Co-ops are at least five to six generations removed from the *M. floribunda* 821 genome. As the resistant bulk containing all Co-ops is used in searching for linked markers, many loosely linked markers will be undoubtedly identified, which will interfere with the process of selecting tightly linked markers to the *Vf* gene. Fortunately, the extent of the introgressed region differs from one individual to another. Thus, it is possible to select individuals having a short introgressed region either left or right of the target gene and then to quickly focus on the short genomic section around the target gene within which tightly linked markers can be identified. In the last step of the "narrow-down" strategy, we reduced the number of Co-ops making up the two resistant sub-bulks, Rb1 and Rb2, to only 1 individual in each, thus narrowing the field of search and maximizing the resolution efficiency. Apart from the introgressed region around the *Vf* gene, Co-op selections may contain some other genetic backgrounds from *M. floribunda* 821. If the two resistant sub-bulks (or 2 individuals in the last step) share the same background noise, falsely linked AFLP bands can be observed that have the same characteristics as those truly linked markers in appearing in *M. floribunda* 821 and Rb (or Rb1 and Rb2) but not in Sb. In fact, many putative AFLP bands have proven to be false, and hence are derived from the background noise.

A rough-mapping using the Co-op material has identified 15 closely linked AFLP markers that are present in every Co-op selection. A high-resolution mapping has shown that all these markers are located within 0.6 cM of the *Vf* region, indicating that the 2 Co-op selections used as Rb1 (Co-op 13) and Rb2 (Co-op 8) contain very short introgressed regions left to and right to the *Vf* gene. Interestingly, nearly half of these 15 AFLP markers are mapped at the same position with AM19/AL07. This may be due to one of the two following factors: (1) suppression of recombination in the region right of the *Vf* gene, which results in a high ratio of physical distance/cM, as alluded to by Patocchi et al. (1999); and (2) the large difference in nucleotide sequence between *M. floribunda* 821 and the cultivated apple (*M.×domestica*) in this region, which will generate more polymorphic bands than other chromosomal regions. Our three resistant populations failed to further resolve our 7 co-segregating AFLP markers. A larger segregating population is necessary to further narrow down the *Vf* region and to facilitate map-based cloning of the *Vf* gene. These 7 markers are highly useful for screening an apple BAC library and constructing a BAC contig around the *Vf* gene, but none of them can serve as starting points in positional cloning as we can not determine which one is located left and/or right of the *Vf* gene. At this point, we can only suggest that the left starting marker will be the one closest to the *Vf* gene, M18 or *ET9MC3–1*, and that the right starting marker will be the one closest to the *Vf* gene, AM19, as also found by Pattochi et al. (1999b), or any of the 7 AFLP markers located at the same site.

A segregant population composed solely of resistant individuals, as in this study, is more reliable than a "normal" population (i.e., containing both resistant and susceptible seedlings) because escape plants can be easily picked out after checking marker data. This problem of escapes has become more evident in our analysis and those of others (Patocchi et al. 1999a; Tartarini 1996). It can be assumed that a resistant individual must have the introgressed segment around the resistance gene; therefore, at least 1 of the closely linked markers flanking the

gene must appear in resistant individuals, otherwise the "resistant" plant must then be an escape. In this study, 2 individuals failed to give rise to any positive bands with any of the tested markers; these are deemed as escapes.

The fine linkage map of the *Vf* gene developed in this study is in agreement with almost all of the maps previously published – Patocchi et al. (1999a, b), Gardiner et al. (1996), King et al. (1998), Gianfranceschi et al. (1996), and Tartarini (1996). However, it is the most saturated map published thus far and therefore is the most useful in map-based cloning of the *Vf* gene. A single recombinant has been found between AM19/AL07/*EA9MC15–1/EA4MG1–1/EA16MG2–1/ET4MC14–1/ET3MG10–2/ET8MG16–1/ET10MG8–1* and *Vf*, and 2 recombinants have been found between M18/*ET9MC3–1* and *Vf*. These findings along with saturation of the *Vf* region with AFLP markers provide a detailed analysis of the *Vf* genomic region that will significantly enhance efforts to identify BAC contigs carrying a large region of the *Vf* gene.

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