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Narrowing down the region of the *Vf* locus for scab resistance in apple using AFLP-derived SCARs

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Abstract A narrow-down strategy to restrict the *Vf* region, which controls resistance to the fungal disease apple scab in apple, to a genetic distance of 0.4 cM is presented. Using 11 AFLP-derived SCARs and three RAPD-derived SCARs, all linked to the *Vf* gene, we subjected 1,412 scab-resistant individuals from 16 mapping populations to genotype analysis. Eleven recombinant individuals were identified within a genetic distance of 0.9 cM around the *Vf* gene. Using these 11 recombinants, we achieved fine-resolution of several AFLP-derived SCAR markers surrounding the *Vf* gene, resulting in the following genetic linkage map: ACS-6 and ACS are located left of the *Vf* gene at genetic distances of 0.2 cM and 0.1 cM, respectively; ACS-7 and ACS-9 are inseparable from the *Vf* gene; ACS-8, ACS-10, and ACS-4 are located to the right of the *Vf* gene at genetic distances of 0.1 cM, 0.4 cM, and 0.5 cM, respectively; the remaining five SCARs—ACS-11, ACS-5, ACS-2, ACS-1, and AL07—are inseparable and are located right of the *Vf* gene at a genetic distance of 0.7 cM. By integrating this linkage data with our previous physical map, we generated a revised map of the narrowed-down region of *Vf*.

Keywords Apple · AFLP · SCAR · Apple scab disease · *Vf* gene

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Introduction

Apple scab attacks both leaves and fruits of apple trees, thereby reducing yield, tree longevity, and fruit quality. The *Vf* gene, originating from the small-fruited crabapple *Malus floribunda* 821, confers resistance to apple scab disease caused by *Venturia inaequalis* (Cke.) Wint. (Crosby et al. 1992) and has been introgressed into large-fruited apple cultivars via sexual hybridization in the Illinois-Purdue-Rutgers apple breeding programs as well as other breeding programs around the world (Crosby et al. 1992; Bus et al. 2002; Zeppa et al. 2002). The *Vf* gene confers resistance to races 1–5 of *V. inaequalis* (Williams and Kuc 1969). It also maintains resistance to race 6 of *V. inaequalis* in *M. floribunda* 821 and in some of its derivatives (Parisi et al. 1993), but it is vulnerable to race 7 (Roberts and Crute 1994). Race 6 has thus far only been identified in European orchards (Bénaouf and Parisi 2000), while race 7 has only been reported in England (Roberts and Crute 1994).

Molecular markers tightly linked to the *Vf* gene have been identified and converted into reliable sequence-specific PCR-based markers, including sequence-characterized amplified regions (SCARs) (Yang and Korban 1996; Yang et al. 1997; Hemmat et al. 1998; Tartarini et al. 1999; Xu et al. 2001) and cleaved-amplified polymorphic sequences (CAPs) (Gianfranceschi et al. 1996). These sequence-specific markers have since been utilized to develop genetic linkage maps (Gardiner et al. 1996; Hemmat et al. 1998; Tartarini et al. 1999) that span the *Vf* region. Recently, Xu et al. (2001) have provided the most reliable map of the *Vf* region using a mapping population consisting of 468 resistant individuals. However, efforts to pursue marker-assisted selection (MAS) and map-based cloning of the *Vf* gene can tremendously benefit from establishing a high-order resolution linkage map of this region.

In the investigation reported here, 1,412 scab-resistant individuals were genotyped using 14 previously developed SCAR markers to narrow down the region of the *Vf* locus, resolve clustering of some SCARs in this region,

and establish a high-order fine-resolution linkage map of the *Vf* gene.

Materials and methods

Plant material

Apple seedling trees (15–20 years old), derived from 16 crosses (Table 1), growing at the pomology farm at the University of Illinois, Urbana, Illinois were used in this study. All seedlings had been previously inoculated in the greenhouse (as young 8- to 10-week-old seedlings) with races 1–5 of *Venturia inaequalis*, and those scab-resistant seedlings were then transferred to the field for fruit evaluation and selection. All of the seedlings carry at least one *Vf* gene derived from one of the parents. In some cases, other scab-resistance genes, *Va* from Antonovka PI 1726623 and *Vr* from *Malus pumila* RI274057A (Williams and Kuc 1969), were present in the second parent and contribute to scab resistance in these seedlings. It is worth noting that it is only the *Vf* gene that confers resistance to all five races of *V. inaequalis* present in North America (Williams and Kuc 1969).

Leaf collection and DNA extraction

Juvenile leaf tissue was collected from field-grown seedling trees, placed into 2.0-ml tubes, labeled, and immediately stored in dry ice while in the field. Samples were then stored at −70°C until needed for DNA extractions.

Apple genomic DNA was extracted from leaf tissue using the protocol previously described by Xu and Korban (2000). Approximately 0.1 g of leaf tissue, frozen in liquid nitrogen, was pulverized with a bamboo skewer, and then ground to a fine powder with steel beads using a high-speed vortex. Following the manufacturer's protocol, DNA was then isolated from the leaf tissue using the Nucleon PHYTOPURE plant DNA extraction kit (Amersham Life Science, Piscataway, N.J.). RNase was added during the incubation step. However, instead of developing a pellet during the centrifugation step, DNA was hooked onto a toothpick out of the isopropanol rinse and subsequently rinsed in 70% ethanol. The DNA was dried and dissolved in TE, and the concentration determined using a UV-VIS spectrophotometer (Shimadzu) was then adjusted to 100 ng/µl for PCR amplification (Xu and Korban 2000).

SCAR markers

A total of 14 SCAR markers linked to the *Vf* gene were used in this study. These SCARs included ACS-1 to ACS-11, OPAL07, S5, and SCAR-OPAR4. Eleven of the SCARs, ACS-1 to ACS-11, are derived from amplified fragment length polymorphism (AFLP) markers and have been previously developed in our laboratory (Xu et al. 2001), while SCARs OPAL07 (Tartarini et al. 1999), S5 (Hemmat et al. 1998), and OPAR4 (Yang et al. 1997) are derived from random amplified polymorphic DNA (RAPD) markers.

Polymerase chain reaction

All PCR reactions were performed in 96-well microtitre plates using a PTC-200 Peltier Thermal Cycler (MJ Research, Waltham, Mass.). The reaction volume of 10 µl contained 1 µl genomic apple DNA (approx. 100 ng), 1 µl 10× PCR buffer, 0.4 µl 50 m*M* MgCl2**,** 1 µl 1 m*M* dNTPs, 1 U *Taq* polymerase (Gibco Life Technologies, Gaithersburg, Md.) and forward and reverse primers of each SCAR marker. The following PCR reaction was used for all ACS SCARs, except for ACS-3: one cycle of 2 min at 94°C; 35 cycles of 30 s at 94°C, 30 s at 65°C, and 2 min at 72°C. For ACS-3, an annealing temperature of 60°C was required. The PCR reactions for all of the other SCARs, OPAL07 (Tartarini et al. 1999), S5 (Hemmat et al. 1998), and OPAR4 (Yang et al. 1997) have been previously published. PCR products were separated on a 1.0% agarose gel with ethidium bromide and visualized under UV light using the Eagle-eye II gel documentation system (Stratagene, La Jolla, Calif.).

SCAR analysis

All 1,412 resistant individuals from all 16 crosses were tested for their genotypes using each of the 14 SCARs. For genotyping of resistant individuals, the PCR banding pattern for each SCAR marker must be consistent with that of the positive control, *M. floribunda* 821. A scab-susceptible cultivar, Rome Beauty, was used as a negative control. Therefore, all SCAR markers should be present in resistant individuals and *M. floribunda*, but absent in cv. Rome Beauty. When a SCAR marker is absent in a resistant individual, then a recombination event must have occurred in the *Vf* region, and this recombinant can then be used for narrowing down the region of the *Vf* locus. To eliminate PCR artifacts and experimental errors and to confirm reliability of the banding profiles, we screened all samples three times with each SCAR marker; i.e., DNA was extracted three times from each individual and subjected to SCAR analysis.

Table 1 Parental genotypes, crosses, and scab resistance

Crosses[a]	Source of resistance[b]	Number of individuals
Coop 17 (1689–110) × Golden Delicious	*Vf*	126
Raritan × Prima	*Vf*	83
Raritan × OB1R1T66	*Vf*	46
Raritan × Coop14	*Vf*	20
Blushing Golden × HCR5T182	*Vf*	59
Coop 13 (2175-7) × NJD2R30T230	*Vf*	55
HCR22T198 (2175-17) × PAR15T1 (2597-100)	*Vf*×*Vf*	161
Golden Delicious × Coop 17 (1689-110)	*Vf*	61
HAR10T156 (1686-4) × OB1R2T56	*Vf*×*Vr*	23
HAR10T156 (1686-4) × TNR10T11	*Vf*×*Vf*	149
NJ63 × HCR22T112	*Vf*	50
Coop 13 × PAR 15T1	*Vf*×*Vf*	101
CCR3T11 × Coop 14	*Va*×*Vf*	10
Co-op Selections (1-38)	*Vf*	38
Co-op 17 × Co-op 16	*Vf*×*Vf*	203
Jonafree × Ill. Del. No.1	*Vf*×*Vf*	227

[a] Female parent is listed first
[b] *Vf* is derived from *Malus floribunda* 821; *Vr* is derived from *M. pumila* R1274057A; *Va* is derived from Antonovka PI 1726623

To develop a high-order fine-resolution map of the *Vf* locus, we adapted the Cri-Map mapping software, CRI-MAP version 2.4 (Green et al. 1990), to process marker data. Map distances were calculated using the mapping function of Kosambi (1944).

Results and discussion

For any successful map-based cloning effort, it is important to accurately narrow-down the region of the target gene by saturating the region with reliable molecular markers (Tanksley et al. 1992). The molecular markers saturating the region of the target gene must result in low rates of recombination so that the genetic distance between the target locus and these markers is relatively short (Hittalmani et al. 2000). To increase the likelihood of successful genotyping of individuals, the marker system must be highly reliable and easily reproducible (Bradeen and Simon 1998; Shan et al. 1999). PCR-based markers, such as SCARs and CAPs, are sequence-specific and are highly reliable in amplifying genomic DNA (Bradeen and Simon 1998).

In this study, both RAPD-derived and AFLP-derived SCAR markers were used to screen all 1,412 resistant individuals. RAPD markers are easy to convert to either SCARs or CAPs for rapid detection as DNA fragments are generally in the size range of 500 bp to 1,500 bp (Barret et al. 1998). However, protocols using RAPD-derived SCARs and CAPs for mapping the *Vf* gene have proven to be of low efficiency. The closest genetic distance mapped using these RAPD-derived SCARs is within 0.2 cM of the *Vf* gene, but none of these SCARs co-segregate with the *Vf* gene (Tartarini et al. 1999). Many AFLP markers are 150–300 bp in size and, consequently, it is necessary to isolate their flanking regions for conversion into SCARs (Bradeen and Simon 1998; Shan et al. 1999). Moreover, it has been reported that AFLP-derived SCAR markers can lose their sequence specificity or their ability to amplify genomic DNA (Schwarz et al. 1999; Shan et al. 1999).

Recently, Xu et al. (2001) have successfully converted 11 out of 15 AFLP markers linked to the *Vf* gene into SCARs. These SCARs have proven to be highly reliable as the linkage map of the *Vf* gene using these SCARs is consistent with its corresponding AFLP map (Xu and Korban 2000; Xu et al. 2001). Based on this previously established high-resolution linkage map (Xu et al. 2001), the genetic distances relative to the *Vf* gene of the following AFLP-and RAPD-derived SCARs are as follows: OPAR4 (0.7 cM) and ACS-6 (0.4 cM) are located left of the *Vf* gene; SCARs ACS-3, ACS-7, ACS-9 co-segregate with the *Vf* gene; SCARs OPAL07, ACS-1, ACS-2, ACS-4, ACS-5, ACS-8, ACS-10, ACS-11 (at 0.2 cM), and S5 (2.0 cM) are located right of the *Vf* gene. This *Vf* linkage map has been developed using a relatively small-sized population of 468 resistant individuals. Therefore, in order to further resolve the location and order of these SCARs along the *Vf* linkage map, a large population of scab-resistant individuals (1,412) was used in this study.

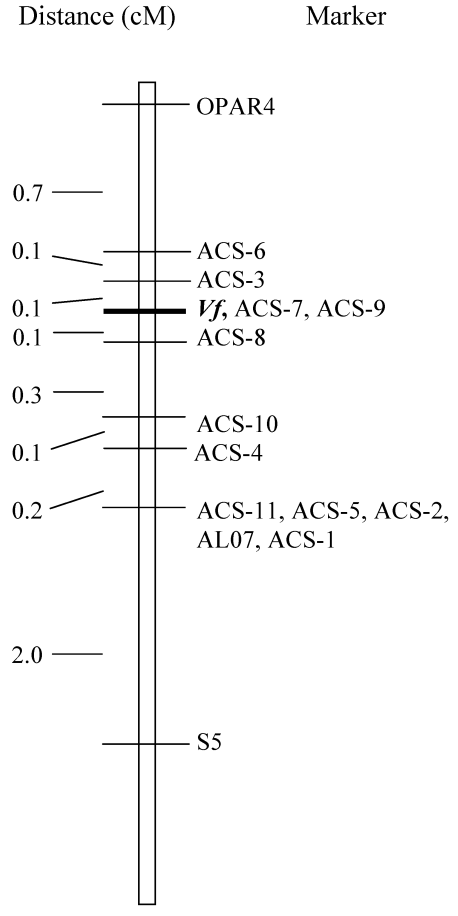

Fig. 1 A high-order fine-linkage map of the *Vf* region using 14 SCAR markers to narrow down the *Vf* locus

The following strategy was used in screening this large population of scab-resistant individuals, derived from 16 apple progenies, carrying the *Vf* gene for scab resistance. Each individual was carefully screened with SCARs to eliminate any escapes and confirm accurate genotyping. The first step in this strategy involved screening all resistant individuals with SCARs S5 (right of *Vf*) and OPAR4 (left of *Vf*) as both of these SCARs are the most remote markers located within the *Vf* region. Secondly, all of the recombinants identified within the interval between OPAR4 and S5 were selected and then screened with ACS-5 (right of *Vf*) and ACS-6 (left of *Vf*). Thirdly, all of the recombinants identified within the interval of ACS-5 and ACS-6 were then screened with SCARs ACS-7 and ACS-9, both co-segregating with the *Vf* gene. Finally, all individuals missing any of the six tested SCAR markers were deemed recombinants and subsequently used in mapping all remaining SCAR markers. As expected, the map constructed in this study (Fig. 1) using a large number of resistant individuals is consistent with our previously published *Vf* linkage map (Xu et al. 2001).

Moreover, a high-order separation of SCAR markers surrounding the *Vf* gene was achieved.

Using a population consisting solely of resistant individuals is highly desirable in rapidly narrowing down the *Vf* locus, as this limits the number of individuals that are genotyped. It is assumed that the resistant individuals retain a large portion of the introgressed *Vf* region. A segregating population consisting of both resistant and susceptible individuals can yield misleading results as errors in phenotyping can play a significant role in data analysis and escape plants can not be readily accounted for (Xu and Korban 2000; Bus et al. 2002). In this study, only 20 individuals were deemed escapes due to their failure in amplifying any of the *Vf*-linked SCARs used in PCR reactions.

We detected a total of 11 recombinants within the *Vf* region. Recombinants were then used to narrow down the donor region and enabled restriction of the *Vf* region (Fig. 1). By identifying these recombinants, we were then able to use these individuals to separate closely linked markers, as recombinants possess various amounts of the introgressed *Vf* region. Of these 11 recombinants, three had a short introgressed region left of the *Vf* gene, while eight recombinants had short introgressed regions of varying amounts right of the *Vf* gene. A single recombinant was found between ACS-6 and ACS-3, two recombinants were found between ACS-3 and *Vf*, two recombinants were found between ACS-8 and *Vf*, two recombinants were found between ACS-10 and ACS-8, two recombinants were found between ACS-4 and ACS-10 (Fig. 2), and two recombinants were found between each of ACS-11, ACS, ACS-2, ACS-1, OPAL07 and ACS-4. These findings resulted in further resolution of these SCAR markers along the *Vf*-linked map, thus narrowing down the *Vf* locus to 0.2 cM.

By integrating these new linkage data with our previous physical map (Xu and Korban 2002a), we then created a revised map (Fig. 3). Based on this new linkage map, ACS-6 is located left of the *Vf* gene at a genetic distance of 0.2 cM; ACS-3 is located left of the *Vf* gene at a genetic distance of 0.1 cM; ACS-7 and ACS-9 are inseparable from the *Vf* gene; ACS-8 is located right of the *Vf* at a genetic distance of 0.1 cM; ACS-10 is located right of the *Vf* gene at a genetic distance of 0.4 cM; ACS-4 is located right of the *Vf* gene at a genetic distance of 0.5 cM; ACS-11, ACS-5, ACS-2, ACS-1, and OPAL07 are located right of the *Vf* gene at a genetic distance of 0.7 cM.

Although several maps have been previously generated using RAPD, RFLP, isozyme, and simple sequence repeat marker systems (Gardiner et al. 1996; Tartarini et al. 1999), the use of AFLP-derived SCAR markers has resulted in the most useful markers in narrowing down the region of the *Vf* gene as reported in this study. Moreover, this revised map (Fig. 3) has allowed us to conduct an analysis of the physical region surrounding *Vf*. A contiguous array of 12 bacterial artificial chromosome (BAC) clones covering the *Vf* region has been identified using these AFLP-derived SCARs. Those SCARs within

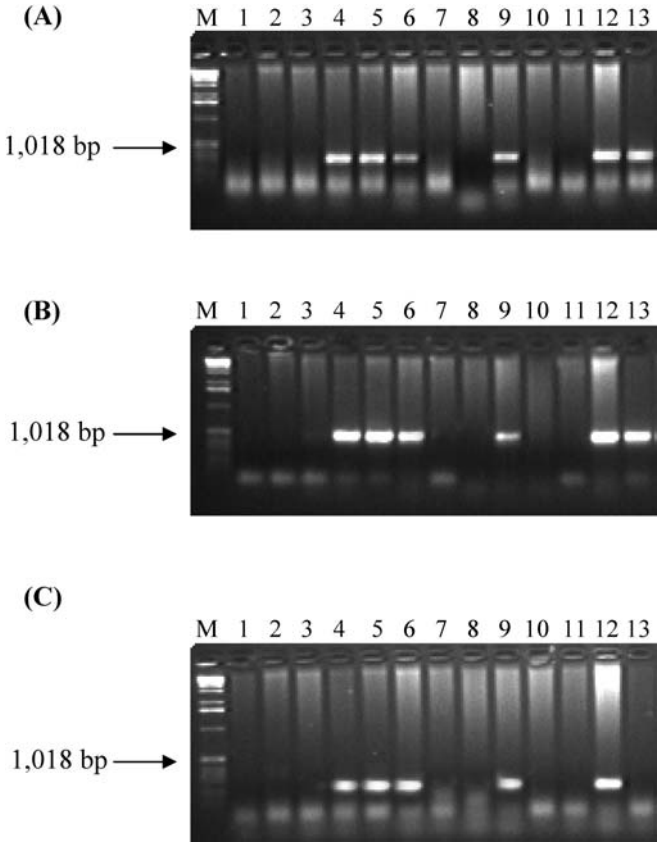

Fig. 2A–C Segregation of apple seedlings for presence/absence of *Vf*-linked SCAR markers. *Lanes*: *M* 1-kb DNA ladder, *4–6*, *9*, *12* resistant individuals amplifying all SCAR markers, *1–3*, *7*, *8*, *10*, *11* resistant individuals deemed escapes, *13* a recombinant with a short introgressed region right of the *Vf* gene between ACS-10 and *Vf*. **A** Amplification with ACS-6 (*left* of the *Vf* gene), **B** amplification with SCAR ACS-7 (co-segregating marker), **C** amplification with SCAR ACS-4 (*right* of the *Vf* gene)

0.2 cM of the *Vf* gene has allowed for the construction of a 140-kb BAC contig consisting of a minimum of three overlapping BAC clones, M4-P11, J11-J23, and J53-N7. These BAC clones have been assembled using SCARs ACS-3 (left of the *Vf* gene), ACS-7 and ACS-9 (co-segregating with *Vf*), and ACS-8 (right of the *Vf* gene).

A fundamental step in map-based cloning is the development of a fine-linkage genetic map as this map can greatly expedite chromosome walking or, better yet, allow direct landing on the target gene (Tanksley et al. 1995). The development of a fine-linkage genetic map often requires several segregating mapping populations in order to avoid marker clustering and problems in over-estimating the ratio of genetic to physical distances (Brunner et al. 2000). With this high-order fine resolution map of the *Vf* gene in hand, we have successfully constructed a megabase-sized BAC contig of the *Vf* region (Xu and Korban 2002a). This has since been followed by a successful positional cloning effort of the *Vf* gene using a chromosome landing strategy that has revealed the presence of a cluster of four receptor-like

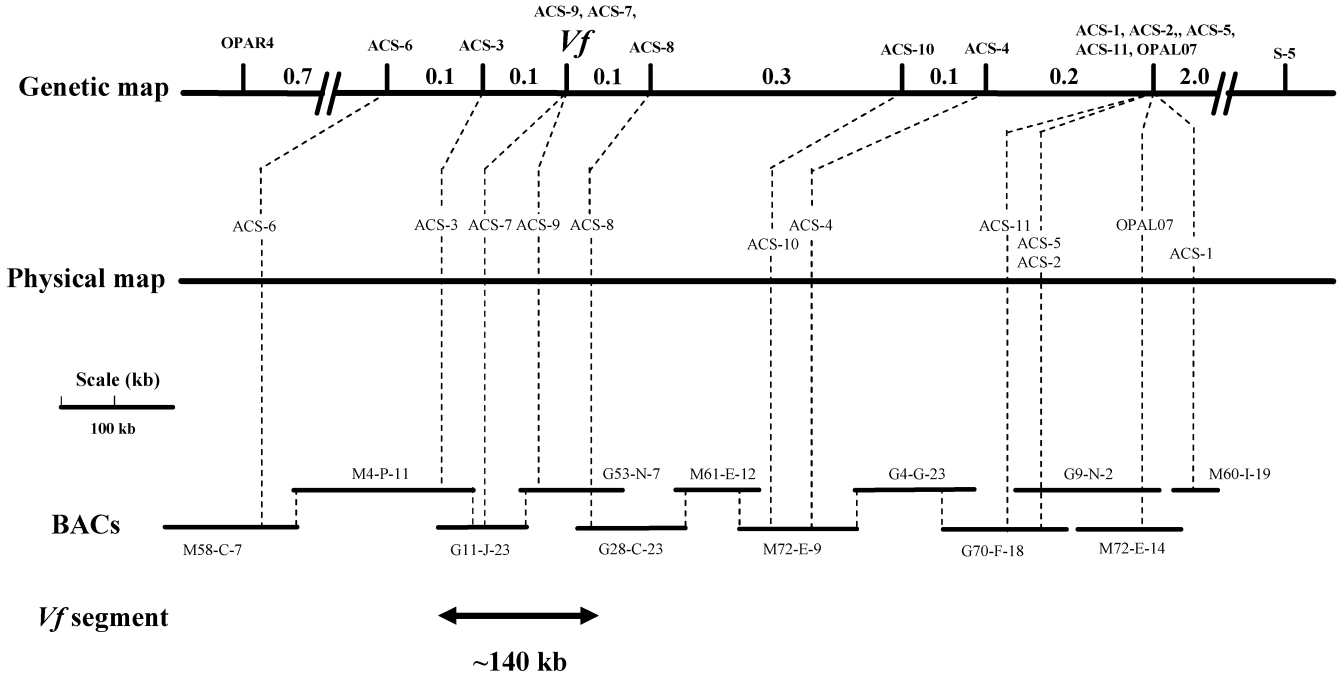

Fig. 3 Integrated genetic and physical maps of the narrowed-down region surrounding *Vf*

gene homologues within the *Vf* locus (Xu and Korban 2002b).

It is anticipated that this high-order resolution map will be also useful in pursuing an efficient marker-assisted breeding scheme for enhancing resistance to apple scab in apple breeding programs.

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