

A genetic linkage map for an apricot (*Prunus armeniaca* L.) BC₁ population mapping plum pox virus resistance

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Abstract Plum pox virus (sharka; PPV) can cause severe crop loss in economically important *Prunus* species such as peach, plum, apricot, and cherry. Of these species, certain apricot cultivars ('Stark Early Orange', 'Goldrich', 'Harlayne') display significant levels of resistance to the disease and are the genetic substrate for studies of several laboratories working cooperatively to genetically characterize and mark the resistance locus or loci for marker-assisted breeding. The goals of the work presented in this communication are the characterization of the genetics of PPV resistance in 'Stark Early Orange' and the development of co-dominant molecular markers for marker-assisted selection (MAS) in PPV resistance breeding. We present the first genetic linkage map for an apricot backcross population of 'Stark Early Orange' and the

susceptible cultivar 'Vestar' that segregates for resistance to PPV. This map is comprised of 357 loci (330 amplified fragment length polymorphisms (AFLPs), 26 simple sequence repeats (SSRs), and 1 morphological marker for PPV resistance) assigned to eight linkage groups. Twenty-two of the mapped SSRs are shared in common with genetic reference map for *Prunus* (T × E; Joobeur et al. 1998) and anchor our apricot map to the general *Prunus* map. A PPV resistance locus was mapped in linkage group 1 and four AFLP markers segregating with the PPV resistance trait, identified through bulk segregant analysis, facilitated the development of SSRs in this region.

Keywords Sharka disease · *Prunus* · Genetic linkage map · SSR · AFLP

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Introduction

Plum pox virus (PPV) causes devastating disease in *Prunus* trees (Ravelonandro et al. 2000). PPV disease, often referred to as sharka disease, affects stone fruits such as apricot, peach, plum, and cherry, which are economically important species of *Prunus*. Originating in Bulgaria in the early 1900s (Atanasoff 1932), PPV has spread throughout Europe (Nemeth 1994) and, as of 1999, has made its way to the Americas.

Detection of PPV in 1999 in Adams County, PA prompted the implementation of an aggressive eradication program to control its spread (Levy et al. 2000). Eradication is an extremely costly way of controlling PPV. Ultimately, the introduction of resistant cultivars of stone fruits into the orchards is the best long-term solution to virus control. Currently, there are two strategies for obtaining resistant cultivars: (1) identify natural resistance present in *Prunus* germplasm and introduce this resistance into commercial cultivars by standard breeding practices, and (2) engineer resistance through application of transgenic technologies. In the first case, selecting for previously identified natural resistance in breeding programs requires screening of individual trees for the resistance phenotype. This labor-intensive time-consuming process drastically slows the progress of breeding programs. However, marker-assisted selection (MAS), based on markers tightly associated with resistance, is a means to substantially streamline the breeding process. In the case of transgenic approaches to engineer resistance, a transgenic plum developed by Scorza et al. (1994) has a durable PPV coat protein-mediated resistance (Hily et al. 2004). Unfortunately, the inability to reliably regenerate transgenic plants with other *Prunus* species hinders the application of transgene-mediated resistance.

Peach is the most genetically characterized *Prunus* species; however, no known natural resistance to PPV has been identified in peach. In contrast, North American apricot cultivars, such as ‘Stark Early Orange’ and ‘Goldrich’, are resistant to PPV (Polák et al. 1995). Resistant apricot cultivars, along with the apricot’s small genome size of 294 Mb (Arumuganathan et al. 1991) and haploid number of $n=8$, facilitate the study of the genetics of PPV resistance. Recently, three genetic maps for apricot have been published (Hurtado et al. 2002; Vilanova et al. 2003a; Lambert et al. 2004). The maps published by Hurtado et al. (2002) and Vilanova et al. (2003a) contain mapped locations for PPV resistance, and both maps are anchored on the general *Prunus* genetic map (Joobeur et al. 1998). This *Prunus* genetic reference map is the comparative map standard for the *Prunus* community and contains anchor markers for integration of genetic information from all *Prunus* species (Aranzana et al. 2003, Dirlewanger et al. 2004). In addition, Lalli et al. (2005) published the first resistance gene map for

Prunus identifying 42 resistance gene containing loci on the general *Prunus* map. The combined reference or resistance map serves as a resource for identification of candidate resistance genes in *Prunus* breeding materials and for utilization of MAS in resistance breeding.

Using ‘Goldrich’ as the donor of resistance, Hurtado et al. (2002) mapped sharka resistance (PPV resistance) as a single locus controlled trait to a linkage group corresponding to linkage group one (G1) of the general *Prunus* map. Using a different donor of resistance, ‘Stark Early Orange’, Vilanova et al. (2003a) similarly mapped resistance to a region of G1; however, it was not clear if the map locations for this resistance co-localized from the different donors. Many hypotheses exist for the number of genes controlling the PPV resistance trait. One hypothesis suggests that a single gene controls the resistance trait (Dicenta et al. 2000; Martinez-Gomez and Dicenta 2000). Moustafa et al. (2001a, b) suggest two genes control the trait, and Guillet-Bellanger and Audergon (2002) suggest three genes control the trait. The research conducted by Vilanova et al. (2003a) is in agreement with the two-gene hypothesis suggested by Moustafa et al. (2001a, b).

The goals of the work presented in this communication are the characterization of the genetics of PPV resistance and the development of co-dominant molecular markers for MAS in PPV resistance breeding strategies. The first goal involves determining the number of genes controlling the resistance trait and whether mapped resistance loci in populations generated from the same donor of resistance but different susceptible cultivars localize to the same region of the genome. The second goal, development of markers for MAS, relies partially upon the first goal. If mapped resistance loci, generated from the same donor of resistance but different susceptible cultivars, localize to the same region of the genome, molecular markers tightly associated with these loci will have great utility in MAS. In order to investigate the genetics of PPV resistance and develop markers for MAS, we constructed the first genetic linkage map for an apricot backcross population segregating for resistance to PPV using ‘Stark Early Orange’ as the resistance donor. This map is primarily comprised of amplified fragment length polymorphism (AFLP) markers. Simple sequence repeat (SSR) markers from the general *Prunus* reference map were chosen to anchor our apricot map to the general *Prunus* map. Four AFLP markers segregating with the PPV resistance trait, identified through bulk segregant analysis (BSA), facilitated the development of SSRs in this region. Due to the co-dominant nature of SSRs along with their high genetic transportability, the development of SSRs associated with PPV resistance in apricot could facilitate the use of MAS in breeding strategies aimed at breeding for natural resistance. Additionally, these markers can enhance fine mapping of this

region and positional cloning of genes that may contribute to PPV resistance.

This map is a valuable tool for locating the regions involved in PPV resistance and the development of molecular markers within these regions. Together with studies of synteny between peach and apricot currently being conducted and the availability of a genome sequence for peach in the near future, this map will speed the genetic studies of PPV resistance and greatly contribute to solving the problem of the spread of PPV in *Prunus*.

Materials and methods

Plant material

An apricot selection LE-3218 ('Stark Early Orange' × 'Vestar') was crossed as a female parent to 'Stark Early Orange' at the Faculty of Horticulture of Mendel University of Agriculture and Forestry in Lednice na Moravě in 1998. 'Stark Early Orange' is resistant to PPV. LE-3218 is susceptible to PPV. Crosses were performed by hand pollination without isolation of flowers after removing of petals and anthers from flower buds. The BC₁ seeds were stratified at 5°C for 3 months and subsequent seedlings were grown in an insect-proof greenhouse. This plant material was used in BSA.

An apricot selection LE-3246 (resistant to PPV; F₁ individual from 'Stark Early Orange' × 'Vestar') was crossed as a female parent to 'Vestar' (susceptible to PPV) at Faculty of Horticulture of Mendel University of Agriculture and Forestry in Lednice na Moravě in 1999. The BC₁ seeds were stratified and the subsequent seedlings were grown in an insect-proof greenhouse. Eighty BC₁ individuals were used to construct the preliminary AFLP genetic map. Due to potential out-crossing, 67 individuals were used to construct the final genetic map.

PPV inoculation and evaluation of PPV infection

When the stems of the seedlings reached about 5 mm thickness, they were inoculated with a chip-bud from a plum infected with the PPV isolate from apricot cultivar Vegama (Marcus strain; Poncarová and Komínek 1998). Seedlings were pruned directly after grafting to promote the growth of the inoculated bud. Plants without sharka symptoms on shoots growing from the inoculum bud and with negative enzyme-linked immunosorbent assay (ELISA) reaction were re-inoculated. PPV infection was evaluated over five consecutive growth periods through visual symptoms and ELISA (Polák et al. 1997). Pruning was performed at the beginning of each growth period to induce vigorous new shoots for symptom scoring. The

plants, in which PPV was not detected by ELISA, were tested by reverse transcription polymerase chain reaction (RT-PCR) using the PPV specific primers P1 and P2 (Wetzel et al. 1991). Plants were classified as resistant if they did not show symptoms and positive ELISA or RT-PCR reaction in the last three growth periods evaluated.

DNA extraction

Genomic DNA was isolated from fresh apricot leaves using the hexadecyltrimethylammonium bromide (CTAB) protocol described by Eldredge et al. (1992). DNA concentrations were measured by a minifluorimeter (TKO100, Hoefer Scientific). Working solutions of genomic DNA at 100 ng/μl in TE buffer (pH 8.0) were prepared for AFLP analysis.

AFLP amplification

AFLP analysis was performed as described by Vos et al. (1995) with a pre-amplification step using primers with one selective base each. *EcoRI* primers were end-labeled with $\gamma^{33}\text{P}$ -ATP (Andotek) and T4 polynucleotide kinase (Promega). PCR products were separated on 6% denaturing polyacrylamide gels, which were pre-run to reach 45°C. Four microliters of samples were loaded on to gels and electrophoresed at 70 V for 2 h and 30 min, vacuum dried, exposed to Biomax MR films (Kodak) for 1–7 days, and developed. Twelve *EcoRI* primers with one selective nucleotide and 23 *MseI* primers with three selective nucleotides were used to amplify AFLP bands. A total of 47 (*EcoRI* or *MseI*) primer combinations were used for the AFLP analysis. AFLP markers were named using the selective nucleotides in the *EcoRI* primer or the selective nucleotides in the *MseI* primer followed by numbers in descending molecular-size order.

Bulk segregant analysis

BSA was performed according to Michelmore et al. (1991). Equal amounts of pre-amplified DNA obtained from ten resistant individuals and ten susceptible individuals, respectively, of the BC₁ population were pooled to constitute the resistant and susceptible bulks (Bulk+, Bulk-). Each bulk represented the alternative phenotypic states of the loci controlling the selected trait. Two bulk samples along with the parents were subjected to AFLP analysis.

Simple sequence repeat development

Cloning and sequencing of AFLP marker fragments

AFLP bands were cut from dry acrylamide gels and soaked in 100 μl of sterile distilled water overnight at 4°C to

extract the DNA according to Wang et al. (2002). The extracted DNA was used as a template for PCR amplification using primers specific for each AFLP marker fragment. The amplification was carried out in a 25- μ l volume containing 5 μ l of extracted DNA, 90 ng of each AFLP marker fragment specific primer, 2 units of *Taq* DNA polymerase (Fisher Scientific), 1 X PCR Buffer (10 mM Tris-HCl pH 9.0, 50 mM KCl), 1.5 mM MgCl₂, 200 μ M each deoxyribonucleotide triphosphate (dNTP). PCR amplifications were programmed for 12 cycles for 30 s at 94°C, 30 s at 65–0.7°C per cycle, 1 min at 72°C and 22 cycles for 30 s at 94°C, 30 s at 56°C, 1 min at 72°C. A 5- μ l aliquot of the PCR reaction was separated by electrophoresis on an agarose gel to confirm the amplification of the expected size fragments. PCR products were ligated into Promega's pGEM®-T Easy Vector and transformed into JM109 cells (Promega) according to the manufacturer's protocol. A minimum of five clones per AFLP marker fragment were sequenced using M13 universal forward and reverse primers and Applied Biosystems Big Dye® Terminator Sequencing Kit and an ABI 377 automated sequencer.

BAC library screening with labeled AFLP marker fragments

DNA was isolated from the clones containing the AFLP marker fragments. Marker fragments were PCR-amplified in a 25- μ l reaction containing 5 μ l of a 1/100 dilution of plasmid DNA, 90 ng of each AFLP marker fragment specific primer, 2 units of *Taq* DNA polymerase (Fisher Scientific), 1 X PCR Buffer (10 mM Tris-HCl pH 9.0, 50 mM KCl), 1.5 mM MgCl₂, 200 μ M each dNTP. PCR amplifications were programmed as specified above. A 2- μ l aliquot of the PCR reaction was separated by electrophoresis on an agarose gel in order to confirm the amplification of fragments of the expected size and DNA concentration. Approximately 10–50 ng of linear DNA was labeled with 15 μ Ci [α -³²P]dCTP using the random priming method (Feinberg and Vogelstein 1983). Labeled DNA probes were purified on Sephadex G25 columns. Labeled AFLP marker fragment probes were used in hybridizations to a BAC library from the apricot cultivar 'Goldrich'. This library consists of 101,376 clones that are arrayed on six 22 cm² Hybond-N+filters (Amersham, Piscataway, NJ, USA; Vilanova et al. 2003b). The library filter set was pre-hybridized for 2 h and hybridized overnight, according to Vilanova et al. (2003b). Radioactive signals were detected autoradiographically using Kodak X-OMAT Blue XB-1 film. In order to confirm the positive BACs, BAC clones identified in the first screening were inoculated into 100 μ l of lysogeny broth (LB)–Chloramphenicol, and incubated at 37°C overnight. BAC clones were stamped onto Hybond-N+filters (Amersham, Piscataway, NJ, USA)

placed on LB–Chloramphenicol agar plates, and incubated overnight at 37°C. The filters were removed from the agar plates and treated with a denaturing solution (1.5 M NaCl, 0.5 M NaOH) followed by a neutralizing solution (1.5 M NaCl, 0.5 M Tris, pH 7.2, 1.0 mM EDTA), rinsed with 2X SSC and the filters were baked at 80°C for 2 h in order to fix the DNA to the filters. Hybridization of these filters and detection of positive BAC clones were carried out as previously described.

Sub-cloning of confirmed positive BACs and identifying and sequencing SSRs

BAC insert DNA was purified for at least one confirmed positive BAC per AFLP marker fragment probe. Purified BAC DNA was digested with *Hind* III and ligated into *Hind* III digested, dephosphorylated p-Bluescript vector (Stratagene, La Jolla, CA, USA), according to Wang et al. (2002). Sub-clones of confirmed positive BACs were selected and incubated in 100 μ l LB/ampicillin overnight at 37°C. Sub-clones were stamped onto Hybond-N+filters (Amersham, Piscataway, NJ, USA), placed on LB/ampicillin agar plates, and incubated overnight at 37°C. Filters were removed from the agar plates and the colonies were denatured, neutralized, and fixed on the filters as previously described (see above). The filters were hybridized with a probe containing (CT)₁₀, (GT)₁₀, and (AGG)₁₀ SSR oligonucleotides. The probes were end-labeled with [γ -³²P]dATP using T4 polynucleotide kinase (Promega) according to the manufacturer's protocol. The hybridizations were carried out at 55°C using the same hybridization conditions previously stated. The filters were washed twice with 2X SSC, 0.1% SDS and exposed to Kodak X-OMAT Blue XB-1 film. Insert DNA of BAC sub-clones identified as containing SSRs was isolated and sequenced as previously described (see above). Primers for eight SSRs were designed from the sequences flanking the SSRs using the online primer design program Primer3 (Rozen and Skaletsky 2000) and purchased from Integrated DNA Technologies (Table 1).

SSR analysis

'Stark Early Orange', 'Vestar' and LE-3246 were screened with 117 SSR primer combinations from Aranzana et al. 2003 and the eight SSR primer combinations developed from the BACs containing the AFLP marker fragments associated with PPV resistance. PCR reactions, electrophoresis, and detection of PCR products for the 'Vestar' \times LE-3246 population were carried out according to conditions specified in Zhebentyayeva et al. (2003). SSR primer combinations revealing polymorphism were screened in the entire mapping population.

Table 1 SSRs derived from apricot BACs containing AFLP marker fragments segregating with PPV resistance

AFLP markers	Apricot BAC clones	SSR markers	Repeat	Primer sequences	Size (bp)
EAA/MCAG8b	007G12 010F06 040L13 165P18	aprigms18	(ct)25	F5'-tct gag ttc agt ggg tag ca-3' R5'-aca gaa tgt gcg ttg ctt ta-3'	210
		aprigms17	(ac)14	F5'-gca gga tea tea aca aat ga-3' R5'-ttc gga act tta cca gtt tca-3'	157
EAG/MCAT14b	246B14	aprigms20	(at)7(ac)4	F5' -tgc ttc ctc tea aac gac aa-3' R5'-agt caa agt caa cca tgc cc-3'	167
		aprigms21	(ac)6(at)7(ac)4	F5'-ggg cct acc ttg att gac gg-3' R5'-agt caa agt caa cca tgc cc-3'	411
		aprigms22	(ct)15	F5'-taa cta gct tcc atc aac cc-3' R5'-ttg caa caa ctg gaa tcc ag-3'	241
		aprigms23	(gt)11	F5'-gct gca gtg gaa ttg cag gg-3' R5'-tag act tct cgg atc att ctc-3'	261
EAT/MCCT10b	021P14 018O08 254J22	aprigms19	(ct)12(ca)11	F5'-ttg cct gct tat gct aac ct-3' R5'-ata aat gct tga tct gca agg-3'	368
ETC/MCCT7b	023M11 069F12 081J15	aprigms24	(ct)11	F5'-atc tgc tct ttc cct cac ct-3' R5'-gat tat ccc tea acc cat cc-3'	326

Linkage analysis

MAPMAKER 3.0

All markers were scored as dominant markers. Loci heterozygous in LE-3246 were used for mapping. A local genetic map was constructed with MAPMAKER/EXP 3.0 software (Lincoln et al. 1992). The mapping analysis was conducted using a 4.0 minimum logarithm of odds (LOD) score and 0.25 maximum recombination frequency (θ). The Kosambi mapping function (Kosambi 1944) was used for calculation of map distances.

Joinmap®3.0

The cross-pollinating population type of the Joinmap®3.0 (Van Ooijen and Voorrips 2001) mapping program was used to establish a genetic map that includes all marker classifications for both 'Vestar' and LE-3246. Chi-square analysis (available in Joinmap®3.0) was used to determine if markers departed significantly from expected Mendelian ratios. Linkage groups were established at a LOD score \geq 4.5 and recombination fraction 0.40. The Kosambi mapping function was used for the calculation of map distances. Criteria for goodness of fit (χ^2) of markers in the map were applied as indicated by Dettori et al. (2001).

Kruskal–Wallis test

Due to the highly skewed segregation ratio observed for the PPV resistance trait, a non-parametric mapping method based on the rank sum test of Kruskal–Wallis was

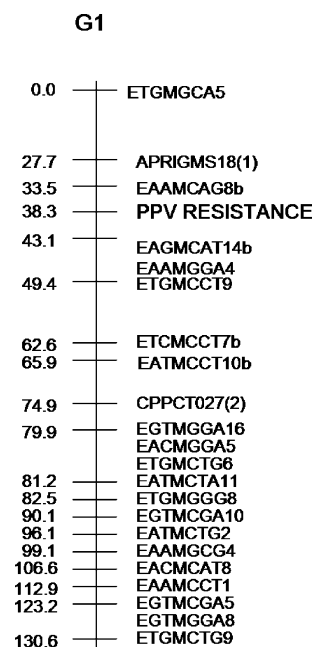
performed on the linkage group G1 data (generated from "Joinmap®3.0", with the MapQTL version 3.0 program (Van Ooijen and Maliepaard 1996)).

Results

PPV segregation analysis

Monogenic, digenic, and trigenic models have been proposed for the number of genes controlling the PPV

Fig. 1 Linkage group G1 genetic map for LE-3246 generated with MAPMAKER 3.0



resistance trait. Moustafa et al. (2000a, b) suggest two genes control the trait and the research conducted by Vilanova et al. (2003a) is in agreement with this two-gene model. Eighty individuals from the LE-3246 × ‘Vestar’ progeny were screened for PPV resistance. Plants were classified as resistant if they did not show symptoms and/or positive ELISA or RT-PCR reaction in the last three growth periods evaluated. Thirteen individuals were found resistant and 67 susceptible. Resistant individuals were coded as heterozygous for the trait and those susceptible were coded as homozygous recessives (consistent with Vilanova et al. 2003a). The segregation ratio 1:5 (resistant/susceptible deviated significantly from the expected for a single dominant locus (1:1) with χ^2 value of 36.5 (resistant/susceptible)). These results clearly indicate that the resistance is controlled by more than a single gene. A PPV resistance locus was mapped in linkage group 1 (Fig. 1) with MAPMAKER 3.0. Several AFLP loci tightly linked to the PPV resistance locus were identified (Table 1).

Bulk segregant analysis

Two hundred and seventy-one primer pair combinations were used to compare the AFLP profiles of ‘Stark Early Orange’ (S), LE-3218 (L), the resistant bulk (+), and the susceptible bulk (-). Plants were identified as resistant by the method previously described (see “PPV segregation analysis”). Eleven AFLP markers (AA/CGT, AA/GCG, AA/GGA, AT/CCT, GA/GGT, GC/CTT, GG/CGA, GT/CTT, TA/GGC, TC/CCT and TG/GCA) were detected only in ‘Stark Early Orange’ and the resistant bulk. All the markers were linked to the gene in the coupling phase. These markers were used in segregation and linkage analysis in the mapping population (LE-3246 × ‘Vestar’). Five AFLP markers (AA/GCG, AA/GGA, AT/CCT, TC/CCT and TG/GCA) detected by BSA were localized on the LE-3246 map. Three markers (GA/GGT, GC/CTT, TA/GGC) were not polymorphic in the LE-3246 × ‘Vestar’ mapping progeny, two markers (AA/CGT, GG/CGA) did

Table 2 SSR markers mapped in LE-3246 x ‘Vestar’ population

SSR marker	Reference	Origin of SSR	Segregation assignment ^a	Linkage group	χ^2
aprigms16	Vilanova et al. (personal communication)	apricot	<hkxhk> ^b	G1	3.1
aprigms18(1)	see Table 1	apricot	<lmxll> ^c	G1	0
aprigms19(3)	see Table 1	apricot	<lmxll>	G8	0.1
aprigms20(1)	see Table 1	apricot	<lmxll>	G3	0.0
aprigms24(2)	see Table 1	apricot	<nnxnp> ^d	G1	4.0*
BPPCT007	Dirlewanger et al. 2002	peach	<lmxll>	G3	0.6
BPPCT008	Dirlewanger et al. 2002	peach	<hkxhk>	G6	0.5
BPPCT009(1)	Dirlewanger et al. 2002	peach	<nnxnp>	G6	6.1*
BPPCT016(3)	Dirlewanger et al. 2002	peach	<hkxhk>	G1	0.8
BPPCT017(1)	Dirlewanger et al. 2002	peach	<nnxnp>	G5	1.3
BPPCT028	Dirlewanger et al. 2002	peach	<hkxhk>	G1	3.5
CPPCT13	Aranzana et al. 2002	peach	<hkxhk>	G5	6.6*
CPPCT26	Aranzana et al. 2002	peach	<hkxhk>	G1	1.6
CPPCT27(2)	Aranzana et al. 2002	peach	<lmxll>	G1	1.0
CPPCT33(2)	Aranzana et al. 2002	peach	<nnxnp>	G7	2.5
EPDCU1775	Howad et al. 2005	almond	<hkxhk>	G4	10.8*
EPDCU3083	Howad et al. 2005	almond	<lmxll>	G3	0.4
EPDCU3117(2)	Howad et al. 2005	almond	<lmxll>	G8	0.0
EPDCU5100(1)	Howad et al. 2005	almond	<nnxnp>	G1	2.3
pchgms003	Sosinski et al. 2000	peach	<efxeg> ^e	G1	7.0
pchgms005(1)	Sosinski et al. 2000	peach	<hkxhk>	G4	3.1
pchgms005(3)	Sosinski et al. 2000	peach	<lmxll>	G4	1.2
pchgms12(2)	Sosinski et al. 2000	peach	<lmxll>	G1	0.1
pchgms47(1)	Sosinski et al. 2000	peach	<lmxll>	G5	0.1
UDA005(1)	Testolin et al. 2000	almond	<lmxll>	G2	2.8
UDP97–402(2)	Cipriani et al. 1999	peach	<lmxll>	G4	0.4

χ^2 : Chi-square (* $P < 0.05$) for expected Mendelian ratio for marker segregations

^a Segregation assignments for Joinmap 3.0

^b <hkxhk> denotes 3:1 or 1:2:1 ratio segregating in both parents

^c <lmxll> denotes 1:1 ratio segregating in the F1 hybrid LE-3246

^d <nnxnp> denotes 1:1 ratio segregating in the recurrent parent ‘Vestar’

^e <efxeg> denotes 1:1:1:1 three allele locus

not work in the mapping progeny and one marker (GT/CTT) remains independent.

Local genetic map for *PPV* resistance locus (gene)

The map consisted of 22 dominant AFLP markers, two dominant SSR markers, and the *PPV* resistance locus and covered a total genetic distance of 130.6 cM. Linkage group 1 that contains the *PPV* resistance locus is depicted in Fig. 1. The marker loci are distributed relatively evenly within this linkage map. None of the markers showed a skewed segregation pattern.

Cloning and sequencing of AFLP marker fragments

The four AFLP marker fragments identified from bulk segregant analysis and segregating with the *PPV* resistance trait were cloned into Promega’s pGEM®-T Easy Vector (Table 1). A minimum of five clones per AFLP marker fragment were sequenced and the sequences

analyzed by inspection. A primary and secondary sequence were identified for the AFLP fragment EAA/MCAT clones and a primary, secondary, and tertiary sequence were identified for the AFLP fragment EAG/MCAT, EAT/MCCT, and ETC/MCCT clones. The primary sequence was assigned to the sequence that was present in a minimum of three out of the five clones sequenced per AFLP fragment. This sequence was chosen for use in all further experiments.

Identification of BAC clones and development of SSR markers from BAC sub-clones

The primary sequence for each of the four AFLP marker fragments was hybridized individually to an apricot BAC library. The results of these hybridizations are depicted in the online supplemental material. The 11 BAC clones were restriction digested with *Hind* III. From inspection of the restriction patterns, two of the four BAC clones that hybridized to AFLP marker fragment EAA/MCAG8b and

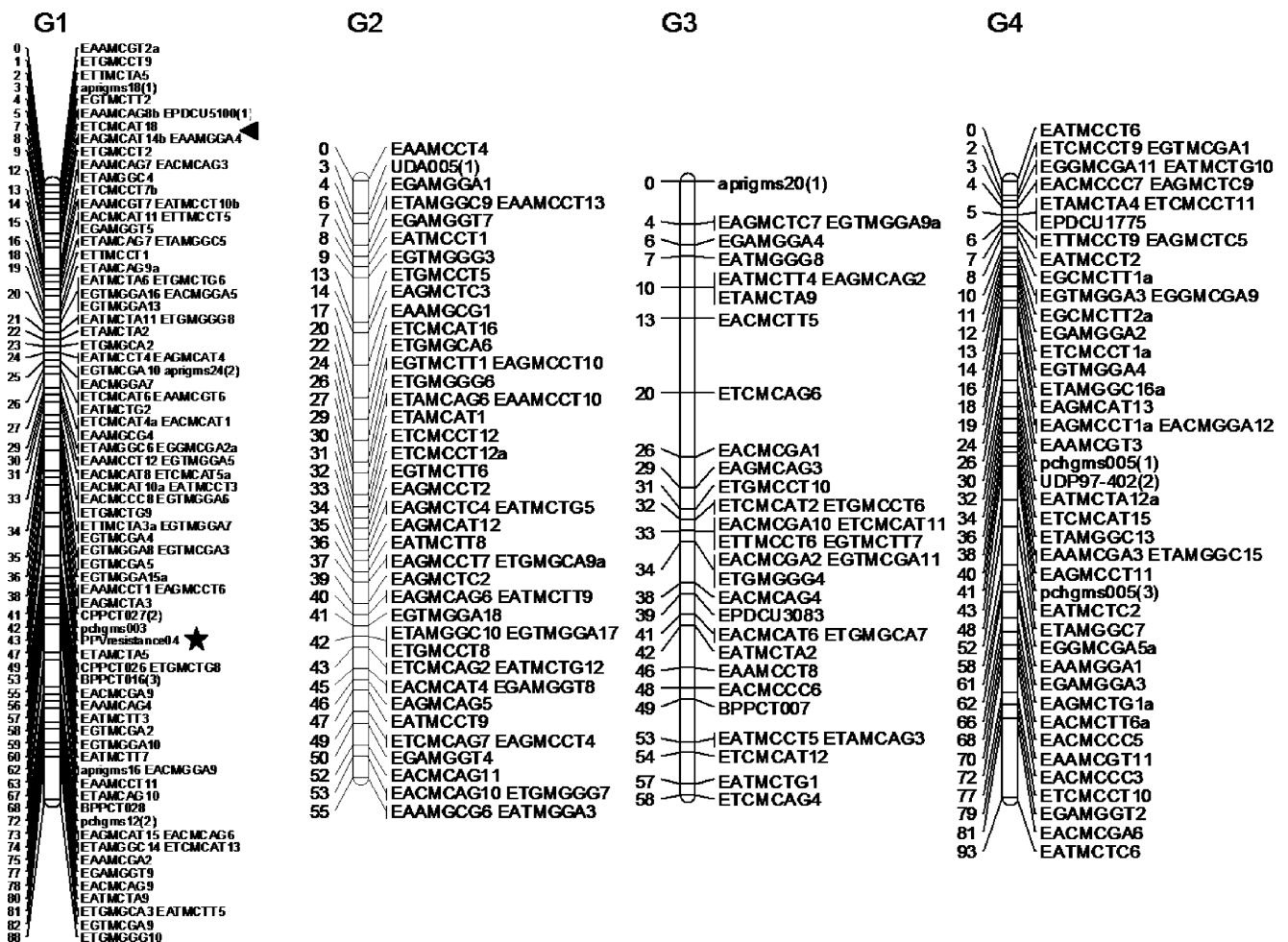


Fig. 2 LE-3246 × ‘Vestar’ map: linkage groups G1, G2, G3, and G4. Genetic linkage map generated with the mapping program Joinmap 3.0. Black arrow indicates the MAPMAKER 3.0 map location for the *PPV* resistance trait. Black star indicates the Joinmap 3.0 map location for the *PPV*

resistance trait. Black arrow indicates the MAPMAKER 3.0 map location for the *PPV* resistance trait

one BAC clone for each of the other AFLP marker fragments were chosen for sub-cloning into p-Bluescript vector. Sub-clones for each of these BACs were colony lifted and hybridized to oligonucleotide probes (CT)10, (GT)10, and (AGG)10. The sub-clone inserts identified as putatively containing SSRs were end-sequenced. Eight SSRs were identified and primers were designed from the flanking sequence for each SSR (Table 1).

AFLP analysis

Forty-seven primer combinations were analyzed in the LE-3246 × ‘Vestar’ mapping population. From this analysis, 423 AFLP markers were used in the construction of the genetic map. One hundred twelve (26.5%) AFLP loci in the F1 hybrid and 112 (26.5%) in the recurrent parent ‘Vestar’ segregated in a 1:1 ratio. One hundred and ninety-nine AFLP loci (47.0%) segregated in both parents in a 3:1 ratio. Deviation ($P < 0.05$) from the expected Mendelian ratio was observed for 74 AFLP loci (17.5%).

SSR analysis

‘Stark Early Orange’, ‘Vestar’, and LE-3246 were screened with 117 SSR primer combinations derived from the general *Prunus* map SSR collection and the eight SSR primer combinations developed from the BACs containing the PPV resistance associated AFLP marker fragments (Table 1). Seventy-five proved to be polymorphic (60%) and were analyzed in the entire mapping population. Only six of the eight SSRs developed from BACs containing the

AFLP marker fragments segregating with PPV resistance were polymorphic. Segregation was demonstrated for 38 of the 75 SSRs (50%) exhibiting polymorphism. These SSRs were used in the construction of the genetic map. Among them, nine segregated in the recurrent parent ‘Vestar’, 18 in the F1 hybrid, and ten in both parents (Table 2). Only one locus with three alleles was detected. Eight SSRs (21%) deviated ($P < 0.05$) from the expected Mendelian ratio.

Linkage map

A total of 450 genetic markers were analyzed for the genetic map construction of the apricot BC1 population (LE-3246 × ‘Vestar’). The map consists of 357 loci (330 AFLPs, 26 SSRs, and one morphological marker for PPV resistance) assigned to eight linkage groups (Figs. 1, 2, and 3). Nomenclature and orientation for the eight linkage groups is assigned according to the genetic reference map for *Prunus* (T × E; Joobeur et al. 1998). Twenty-two of the SSRs placed on the map anchor this map to the genetic reference map for *Prunus*. The average number of loci per linkage group is 45. The range of the number of loci per linkage group is 19 (G5) to 96 (G1). The map covers 522.69 cM of the apricot genome. The range of the length of each linkage group is 51.48 (G8) to 87.90 cM (G1). Linkage group G1 has the highest marker density with an average distance of 0.92 cM per marker and the least saturated linkage group G5 with an average of 3.03 cM per marker. Four gaps (>10 cM) ranging from 10.1 to 13.74 cM were observed in this map.

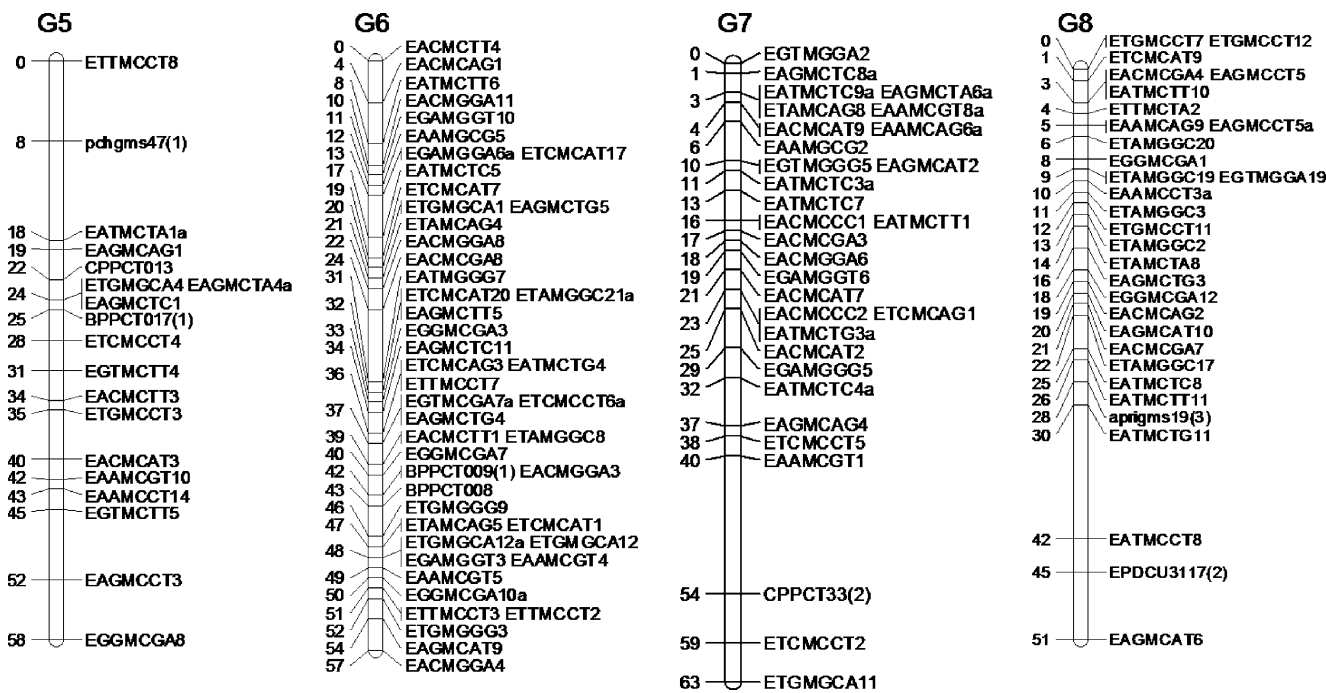


Fig. 3 LE-3246 × ‘Vestar’ Map: Linkage groups G5, G6, G7, and G8. Genetic linkage map generated with the mapping program Joinmap 3.0

Table 3 Distorted loci ($P < 0.05$) mapped in the LE-3246 × ‘Vestar’ apricot BC1 population linkage groups G1, G2, and G4

G1		G2		G4	
Marker	Map distance (cM)	Marker	Map distance (cM)	Marker	Map distance (cM)
ETCMCAT18	7.162	EAGMCTC3	14.215	EACMCCC7	3.992
ETAMCAG9a	19.148	EGTMCTT1	23.815	EPDCU1775	5.237
EATMCCT4	23.876	ETCMCCT12a	30.682	ETTMCTT9	5.607
Aprigms24(2)	24.647	EATMCTG5	33.813	ETCMCCT1a	13.110
ETCMCAT4a	27.341	EATMCTT8	35.617	EGTMGGA4	13.503
EGGMCGA2a	29.297	ETGMGCA9a	37.400	ETAMGGC16a	15.776
EAAMCCT12	29.670	EGTMGGA18	41.496	EAGMCAT13	17.578
ETCMCAT5a	31.264	EGTMGGA17	41.813	EAGMCCT1a	18.936
EACMCAT10a	32.586			EACMGGA12	19.157
ETTMCTA3a	33.650			ETCMCAT15	33.689
EGTMGGA7	33.837			EGGMCGA5a	52.018
PPV RESISTANCE	43.161			EAGMCTG1a	62.497
EGTMCGA2	58.348				
EACMCAG6	72.610				

Fifty-nine of the 357 mapped loci (16.53%) exhibited distorted segregation and are distributed in the map as follows: G1 (14 loci), G2 (8 loci), G3 (0 loci), G4 (12 loci), G5 (1 loci), G6 (13 loci), G7 (8 loci), and G8 (three loci; Tables 3 and 4).

Kruskal–Wallis test

Several hypotheses for the number of genes controlling the PPV resistance trait exist (Dicenta et al. 2000; Martinez-Gomez and Dicenta 2000; Moustafa et al. 2001a, b; Guillet-Bellanger and Audergon 2002). Additionally, the segregation ratio (1:5, resistant/susceptible) of PPV resistance in the LE-3246 × ‘Vestar’ mapping progeny deviates significantly from the single dominant gene segregation ratio of 1:1. Therefore, the Kruskal–Wallis test was performed for all markers of the LE-3246 × ‘Vestar’ linkage map. Only markers present in the upper half of linkage group G1 showed significance levels, $P < 0.005$ for the Kruskal–Wallis test statistic (Table 5; Fig. 4; see “Supplementary material”). Eleven markers showed significance levels, $P < 0.00001$. This suggests that a putative quantitative trait locus (QTL) for the PPV resistance trait may reside in the region of G1 between 1.5 and 40.9 cM.

Discussion

We used AFLP markers to generate a framework genetic map for characterizing PPV resistance in the apricot BC1 population LE-3246 × ‘Vestar’. *EcoRI* primers with one

selective nucleotide and *MseI* primers with three selective nucleotides were used to amplify AFLP fragments. A total of 423 AFLP polymorphic fragments were identified with 17.5% of the AFLP markers showing distorted segregation

Table 4 Distorted Loci ($P < 0.05$) mapped in the LE-3246 × ‘Vestar’ apricot BC1 population. Linkage groups G5, G6, G7, and G8

Linkage group	Marker	Map distance (cM)
G5	CPPCT013	22.272
G6	EACMCTT4	0.000
	EATMCTT6	7.749
	EACMGGA11	10.252
	EGAMGGT10	11.089
	EAGMCTG5	20.190
	EACMGGA8	21.658
	EATMGGA7	31.094
	ETTMCTT7	36.477
	EGTMCGA7a	36.570
	BPPCT009(1)	41.652
	ETGMGCA12a	47.503
G7	ETGMGCA12	47.781
	EGGMCGA10a	50.953
	EAGMCTC8a	1.128
	EATMCTC9a	2.923
	EAGMCTA6a	2.937
	EAAMCGT8a	3.377
	EACMCAT9	3.968
	EAAMCAG6a	4.071
	EATMCTC3a	10.700
	EATMCTG3a	23.449
G8	EAAMCAG9	5.201
	EAGMCCT5a	5.211
	EAAMCCT3a	10.288

Table 5 Kruskal–Wallis statistic adjusted for ties (K*) for those markers located at the LE-3246 x ‘Vestar’ map linkage group G1

Map (cM)	Locus	Nr	K*	Df	SI (P)
0.0	EAAMCGT2a	60	0.003	1	
0.3	ETGMCCT9	65	9.378	1	****
1.5	ETTMCTA5	64	20.529	1	*****
3.6	aprigms18(1)	63	10.667	1	****
3.7	EGTMCTT2	58	1.734	1	
4.8	EPDCU5100(1)	64	9.956	1	****
6.2	EAGMCAT14b	61	8.242	1	****
6.3	EAAMGGA4	62	12.578	1	*****
6.5	ETCMCAT18	60	6.472	1	**
8.7	EAAMCAG8b	63	10.010	1	****
8.8	ETGMCCT2	62	3.197	1	*
11.2	EACMCAG3	66	29.582	1	*****
11.8	EAAMCAG7	63	4.133	1	**
12.5	ETAMGGC4	65	2.113	1	
13.2	ETCMCCT7b	62	11.715	1	*****
13.8	EAAMCGT7	60	6.051	1	**
14.2	EACMCAT11	49	21.273	1	*****
14.4	EATMCCT10b	63	10.333	1	****
14.8	ETTMCTT5	65	6.857	1	***
15.4	EGAMGGT5	53	5.494	1	**
15.7	ETAMCAG7	65	0.530	1	
16.0	ETAMGGC5	65	8.286	1	****
17.6	ETTMCTT1	63	5.072	1	**
19.2	ETAMCAG9a	66	6.258	1	**
19.8	EATMCTA6	55	13.435	1	*****
20.3	ETGMCTG6	54	13.250	1	*****
20.5	EGTMGGA16	67	14.265	1	*****
20.6	EACMGGA5	51	8.788	1	****
20.8	EGTMGGA13	66	21.888	1	*****
21.5	EATMCTA11	56	8.934	1	****
21.7	ETGMGGG8	63	9.667	1	****
22.4	ETAMCTA2	58	2.450	1	
22.9	ETGMGCA2	58	16.394	1	*****
23.9	EATMCCT4	62	4.903	1	**
24.6	aprigms24(2)	64	5.400	1	**
24.6	ETCMCAT6	64	17.732	1	*****
24.8	EAGMCAT4	59	11.043	1	****
25.1	EGTMCGA10	63	8.941	1	****
25.3	EAAMCGT6	60	13.729	1	*****
26.1	EACMGGA7	51	19.793	1	*****
26.4	EATMCTG2	59	6.963	1	***
27.3	ETCMCAT4a	64	8.609	1	****
27.4	EACMCAT1	65	20.779	1	*****
27.9	EAAMGCG4	59	9.003	1	****
28.5	ETAMGGC6	65	21.460	1	*****
29.2	EGGMCGA2a	63	11.907	1	****
29.4	EAAMCCT12	65	28.160	1	*****
31.0	EGTMGGA5	63	4.192	1	**
31.2	ETCMCAT5a	64	8.609	1	****
31.4	EACMCAT8	67	5.980	1	**
32.5	EACMCAT10a	67	8.576	1	****
33.2	EACMCCC8	66	0.228	1	
33.4	EATMCCT3	63	0.015	1	
33.6	ETTMCTA3a	60	6.384	1	**
34.2	EGTMGGA7	63	0.085	1	
34.2	EGTMCGA4	61	0.100	1	

Table 5 (continued)

Map (cM)	Locus	Nr	K*	Df	Sl (P)
34.3	EGTMGGA8	64	5.354	1	**
34.3	EGTMCGA5	61	4.825	1	**
34.5	EGTMGGA6	63	3.010	1	*
36.3	EGTMGGA15a	67	16.150	1	*****
36.3	EGTMCGA3	61	2.738	1	*
36.7	ETGMCTG9	57	4.906	1	**
38.0	EAGMCCT6	65	13.592	1	*****
38.2	EAAMCCT1	65	6.717	1	***
38.6	EAGMCTA3	53	0.049	1	
40.9	CPPCT027(2)	66	8.044	1	****

NR Number of individuals, *Df* degrees of freedom, *Sl* significance levels
 p*=0.1, *p*=0.05, ****p*=0.01, *****p*=0.005, ******p*=0.0001, ******p*=0.0005, ******p*=0.00001

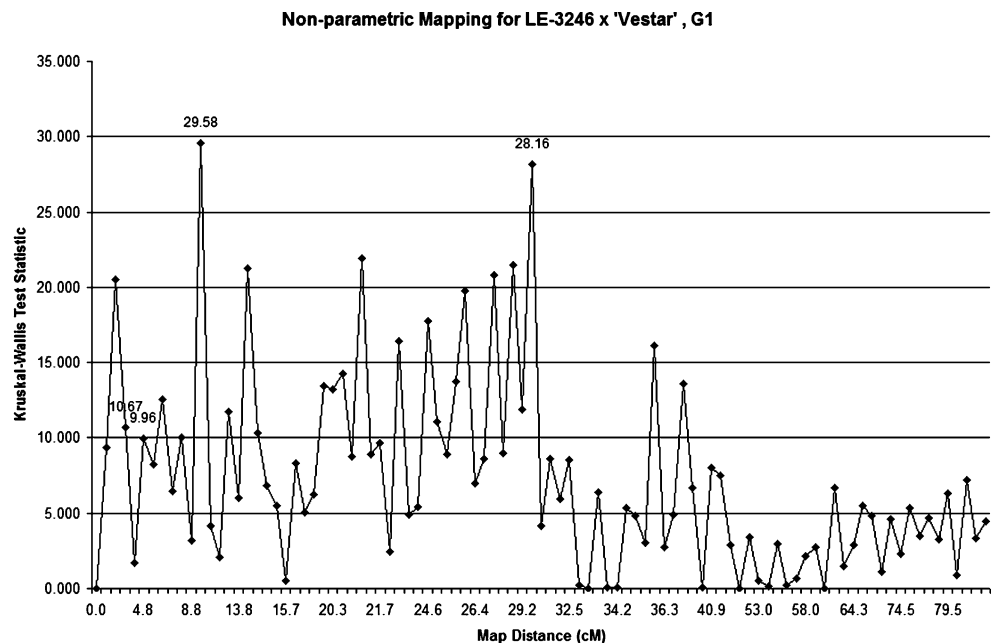
ratios in the progeny. This percentage of distorted markers is slightly higher than the published work in apricot by Vilanova et al. (2003a; 17%), Hurtado et al. (2002; 12%), and Lambert et al. (2004; 10.2%). Distribution of AFLP markers in this map is fairly uniform. Clustering of AFLP markers in centromeric regions was not observed as reported in tomato by Haanstra et al. (1999).

SSR marker detection with primers developed from peach, apricot, and almond (Sosinski et al. 2000; Cipriani et al. 1999; Testolin et al. 2000; Aranzana et al. 2002) demonstrates the high transportability of SSRs in *Prunus* (Dirlewanger et al. 2002). ‘Stark Early Orange’, ‘Vestar’, and LE-3246 were screened with 125 SSR primer combinations. Sixty percent of these SSRs were polymorphic. This percentage of polymorphism is slightly higher than that previously reported by Hurtado et al. (2002) and Vilanova et al. (2003a; 46% and 42%, respectively) in

apricot. However, 12.5% of the SSRs primers detecting polymorphism were derived from apricot and may account for this difference.

We identified four AFLP marker fragments as segregating with PPV resistance through bulk segregant analysis. SSR primers aprigms 17–24 were developed from BACs containing the AFLP fragments segregating with PPV resistance (Table 1). Two of these SSR primers, aprigms 18 and aprigms 24, detected polymorphisms that map to G1. This work presents the first genetic map based on an apricot BC₁ population. It consists of 357 loci (330 AFLPs, 26 SSRs, and 1 morphological marker for PPV resistance) assigned to eight linkage groups (Figs. 1, 2, and 3). Since 22 of the mapped SSRs are shared in common with genetic reference map for *Prunus* (T × E; Joobeur et al. 1998), we employed the nomenclature and orientation for the eight linkage groups of this reference map. The map covers

Fig. 4 Kruskal–Wallis statistic adjusted for ties for those markers located in the LE-3246 × ‘Vestar’ map linkage group G1



522.7 cM of the apricot genome with an average distance of 1.46 cM/marker. The length of the map is consistent with the apricot maps published by Hurtado et al. (2002), Vilanova et al. (2003a), and Lambert et al. (2004) and the average distance per marker is less than all other maps published for apricot (Hurtado et al. 2002; Vilanova et al. 2003; Lambert et al. 2004).

Approximately 17% of the mapped loci exhibited distorted segregation with G1 and G6 exhibiting the largest number of distorted loci. Vilanova et al. (2003a) observed similar results in apricot.

A PPV resistance locus was mapped in linkage group 1 (Fig. 1) with MAPMAKER3.0. PPV resistance also mapped to linkage group G1 using the Joinmap3.0 program; however, the map locations are not the same (Fig. 1). The location for PPV resistance mapped in Joinmap for the BC1 population for LE-3246 × ‘Vestar’ (Fig. 3) and the location tentatively placed in the ‘Lito’ × ‘Lito’ F2 populations appear to be the same location (Vilanova et al. 2003a). Additionally, Romero et al. (2005) performed the Kruskal–Wallis test for mapping PPV as a putative QTL in the ‘Lito’ × ‘Lito’ population. Markers in the upper half of linkage group G1 were significant for the Kruskal–Wallis test statistic (between 12 and 38.4 cM). The same test was performed for all markers of the LE-3246 × ‘Vestar’ linkage group G1. Markers present in the upper half of linkage group G1 were significant for the Kruskal–Wallis test statistic ($P < 0.005$; between 0.3 and 40.9 cM; Table 5; Fig. 4; see “Supplementary material”). The highest level of significance was detected in the same region in both mapping populations and indicates that a putative locus controlling PPV resistance may exist in this region. The most interesting observation is that the non-parametric mapping location for PPV resistance in ‘Lito’ × ‘Lito’ (Romero et al. 2005) and LE-3246 × ‘Vestar’ is very similar to the mapped location for PPV resistance in the LE-3246 × ‘Vestar’ progeny map constructed with MAPMAKER 3.0 (Fig. 1). Additionally, two SSRs, aprigms18(1) and EPDCU5100(1), were significant for the Kruskal–Wallis test statistic and may be useful for MAS for breeding for PPV resistance. Two different strategies employed to map the PPV resistance trait have generated two unique map locations that may putatively represent two genes controlling the PPV resistance trait. Further evaluation of these loci will be necessary to characterize the genetic control of the PPV resistance trait.

PPV resistance is a complex trait controlled by at least two genes. In an effort to characterize PPV resistance, we constructed the first genetic linkage map for an apricot backcross population segregating for resistance to PPV. The map is comprised of AFLP markers along with SSR markers from the general *Prunus* reference map that

anchor our apricot map to the general *Prunus* map. This also facilitates the comparison of our map with other genetic maps for *Prunus*. Four AFLP markers segregating with the PPV resistance trait, identified through BSA, facilitated the development of SSRs in this region. Due to the co-dominant nature of SSRs along with their high genetic transportability, the development of SSRs associated with PPV resistance in apricot could facilitate the use of MAS in breeding strategies aimed at breeding for natural resistance. Additionally, these markers can serve as landmarks to aid in fine mapping of this region as well as enable positional cloning of genes that may contribute to PPV resistance. Mapping the PPV resistance trait and the development of molecular markers associated with trait will be extremely valuable for identifying candidate resistance genes when the genome sequence for peach becomes available.

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