Molecular Genetic Mapping of the Plum Pox Virus Resistance Genes in Apricot

D. Abernathy, T. Zhebentyayeva and A.G. Abbott
Dept. of Genetics and Biochemistry, 100 Jordan Hall, Clemson University, Clemson, SC 29631 USA

S. Vilanova and M.L. Badenes
Instituto Valenciano de Investigaciones Agrarias Apartado Oficial, 46113 Moncada Valencia Spain

J. Salava and J. Poláč
Division of Plant Medicine, Research Institute of Crop Production, Drnovská 507, 161 06 Prague 6 Czech Republic

B. Krška
Faculty of Horticulture Mendel University of Agriculture and Forestry in Brno 691 44 Lednice na Moravě Czech Republic

V.D. Damsteegt
USDA/ARS Foreign disease-Weed Science Research Unit, 1301 Ditto Avenue, Fort Detrick, MD 21702 USA

Keywords: Prunus armeniaca L., molecular markers

Abstract
Plum pox virus (PPV) is a devastating disease of Prunus trees such as apricot, peach, plum, nectarine and cherry. It is the most economically important disease of fruit crops in Europe and as of 1999 has found its way into the United States. Development of molecular genetic markers associated with disease resistance in Prunus species could prove to be a valuable tool in combating disease caused by PPV. In order to do this, a molecular genetic approach has been initiated to map putative resistance regions associated with resistance in apricot. Four dominant AFLP markers segregating with resistance to PPV have been previously mapped in the crosses ‘Stark Early Orange’ (resistant) x ‘LE-3218’ (a susceptible selection) and ‘Vestar’ (susceptible) x ‘LE-3246’ (a resistant selection). The markers EAAJMCAG, EAGIMCAT, EATIMCCI, and ETC/MCCF are located 45, 4.5, 133, and 16.4 cM respectively from the putative resistance gene. In order to further characterize this region, each marker was cloned, sequenced and hybridized to an apricot BAC library. Eleven apricot BACs were positively identified as containing a specific AFLP marker fragment. Based on Hind III digestion patterns of these BACs, 6 of the 11 BACs were chosen for sub-cloning and hybridized to the SSR (simple sequence repeats) oligonucleotide probes. Sub-clones that hybridized to the oligonucleotide probes were sequenced in order to develop SSRs in this region. Eight SSRs identified are currently being investigated to determine their potential for mapping and screening in other crosses. Due to the co-dominant nature of SSRs and their high genetic transportability, development of SSRs linked to resistance to PPV in apricot could allow the plant breeder to use MAS (marker assisted selection) to screen resistant cultivars in apricot and potentially in other Prunus species.

INTRODUCTION
Apricots are the third most important species of stone fruits. World production in 2002 was 2.7 million metric tons (FAO, 2003). Despite its economic importance, apricots are the least genetically characterized species of the genus Prunus (Hurtado et al., 2002). Plum pox virus (PPV) is the cause of devastating disease in Prunus trees
(Ravelonandro et al., 2000) such as apricot, peach, plum, nectarine and cherry. It first originated in Bulgaria and through grafting and aphid transmission has spread its way westward through Europe and recently to the Americas (Nemeth, 1994). In 1999 PPV was detected in Pennsylvania, USA (Levy et al., 2000), and the spread of the virus is being controlled by an aggressive eradication program. Eradication is very costly to the grower and in the end to the consumer. PPV's potential for crippling the American fruit tree industry has prompted the federal government to list it as one of the top ten significant threats to agriculture in the US (see Federal Government ACT: Public Health Security and Bioterrorism Act of 2002, described at http://www.aphis.usda.gov/ppv/permits/agri_bioterrorism).

In order to sustain the fruit crop industry and control the spread of PPV, it will be critical to introduce resistant cultivars into the orchards; however, the time consuming process of screening cultivars for resistance to PPV is the bottle neck in current breeding programs. We have begun developing molecular genetic markers associated with resistance to PPV in apricot. Four dominant AFLP markers segregating with resistance to PPV have been previously mapped in the crosses ‘Stark Early Orange’ (resistant) × ‘LE-3218’ (a susceptible selection) and ‘Vestar’ (susceptible) × ‘LE-3246’ (a resistant selection). The markers EAA/MCAG, EAG/MCAT, EAT/MCCT, and ETC/MCCT are located 4.5, 4.5, 13.3, and 16.4 cM respectively from the putative resistance gene (Salava et al., 2002a, 2002b). In this paper we present the conversion of the AFLP markers EAA/MCAG, EAG/MCAT, EAT/MCCT, and ETC/MCCT into co-dominant SSR (simple sequence repeats) markers and the results of the screening of these markers in three different apricot populations.

MATERIALS AND METHODS

DNA Material

DNA from ‘Stark Early Orange’, ‘Vestar’, and ‘LE-3246’ (a F1 individual from ‘Stark Early Orange’ × ‘Vestar’) and from a back cross population from ‘Vestar’ × ‘LE-3246’ was provided by the Division of Plant Medicine, Research Institute of Crop Production, Prague, Czech Republic (Salava et al., 2002a, 2002b). DNA from ‘Goldrich’, ‘Valenciano’, and F1 population of ‘Goldrich’ × ‘Valenciano’, ‘Stark Early Orange’, ‘Thyrintos’, ‘Lito’ (a F1 progeny of ‘Stark Early Orange’ × ‘Thyrintos’), and a F2 population derived from a self-pollination of ‘Lito’ was provided by the Instituto Valenciano de Investigaciones Agrarias, Valencia, Spain (Hurtado et al., 2002; Vilanova et al., 2003).

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 template for PCR (polymerase chain reaction) amplification using pre-amplification primers specific for each AFLP marker fragment. The amplification was carried out in a 25 μl volume containing 5 μl of extracted DNA, 90 ng of each AFLP marker fragment specific primer, 2 units of Taq DNA polymerase (Fisher Scientific), 1X PCR Buffer (10 mM Tris-HCl pH 9.0, 50 mM KCL), 1.5 mM MgCl2, 200 μM each dNTP. PCR amplifications were programmed for 12 cycles for 30 sec at 94°C, 30 sec at 65°C – 0.7°C per cycle, 1 min at 72°C and 22 cycles for 30 sec at 94°C, 30 sec at 56°C, 1 min at 72°C. A 5 μl aliquot of the PCR reaction was separated by electrophoresis on an agarose gel to confirm the amplification of fragments of the expected size. 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 5 clones were sequenced using M13 universal forward and reverse primers and Applied Biosystems Big Dye® Terminator Sequencing Kit and an ABI 377 automated sequencer.
DNA was isolated from the clones containing the AFLP marker fragments. Marker fragments were PCR amplified in a 25 µl reaction containing 5 µl 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), 1X PCR Buffer (10 mM Tris-HCl pH 9.0, 50 mM KCl), 1.5 mM MgCl₂, 200 µM each dNTP. PCR amplifications were programmed as specified above. A 2 µl 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 µCi [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 variety ‘Goldrich’. This library consists of 101,376 clones which are arrayed on six 22 cm² Hybond-N+ filters (Amersham, Piscataway, N.J.) (Vilanova, et al., 2003). The library filter set was pre-hybridized for two hours and hybridized overnight according to Vilanova et al. (2003) 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 obtained from the BAC library, inoculated into 100 µl of LB/ Chloramphenicol, and incubated at 37°C overnight. BAC clones were then stamped onto Hybond-N+ filters (Amersham, Piscataway, N.J.) 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 two hours 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 digested, dephosphorylated p-Bluescript vector (Stratagene, La Joya, CA) according to Wang et al. (2002). Sub-clones of confirmed positive BACs were selected and incubated in 100 µl LB/ampicillin overnight at 37°C. Sub-clones were then stamped onto Hybond-N+ filters (Amersham, Piscataway, N.J.), 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. The filters were hybridized with a probe containing (CT)₉, (GT)₉, and (AGG)₁₀ SSR oligonucleotides. The probes were end-labeled with [γ³²P]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 (simple sequence repeats) was isolated and sequenced as previously described. Primers for eight SSRs were designed from the sequences flanking the SSRs using the online primer design program Primer3 (Rozen and Skaltsky, 2000) and purchased from Integrated DNA Technologies.

SSR Screening of Three Populations Segregating for PPV Resistance

‘Vestar’ × ‘LE-3246’, ‘Lito’ × ‘Lito’, and ‘Goldrich’ × ‘Valenciano’ populations were screened with eight different primer combinations for apricot SSR loci. PCR reactions and electrophoresis for ‘Vestar’ × ‘LE-3246’ population were carried out according to conditions specified in Zhebentyayeva et al. (2003). For the ‘Lito’ × ‘Lito’ and the ‘Goldrich’ × ‘Valenciano’ populations PCR reactions were carried out in a 20 µl volume containing 20 ng of genomic DNA, 1 X PCR Buffer, 1.5 mM MgCl₂, 200 µM of each dNTP, 10 pmol of each primer, 1.5 units of Taq DNA polymerase. PCR reactions for ‘Lito’ × ‘LE-3246’ and ‘Goldrich’ × ‘Valenciano’ populations were separated by electrophoresis on 2.5% or 3% agarose gels or on 5% or 6% acrylamide gels and stained.
with ethidium bromide.

RESULTS

Cloning and Identification of AFLP Marker Fragments

The four AFLP markers segregating with PPV resistance in the ‘Vestar’ × ‘LE-3246’ population, were cloned into Promega’s pGEM™-T Easy Vector and a minimum of five clones per AFLP marker were sequenced and analyzed manually. For the EAA/MCAG clones, a primary and secondary sequence was identified and for the EAG/MCAT, EAT/MCCT and ETC/MCCT clones, a primary, secondary and tertiary sequence was identified. A minimum of 3 clones sequenced per AFLP marker contained the primary sequence and this sequence was used in all further experiments (see Table 1).

Identification of BAC Clones

AFLP marker fragment probes were hybridized to the apricot BAC library and in the first screening a total of 69 BAC clones were identified as containing a specific AFLP marker fragment. Of the total 69 BAC clones, 28, 17, 13, and 11 BAC clones were identified as containing the AFLP marker fragments associated with the primer combinations EAA/MCAG, EAG/MCAT, EAT/MCCT and ETC/MCCT respectively. However, in the second screening, only a total of 11 apricot BACs were identified (see Table 1). Apricot BACs confirmed in the second screening were digested with Hind III and for each of the four AFLP marker probes, at least one confirmed positive BAC was sub-cloned.

Identification of Sub-clones Containing SSRs and Development of SSR Primers

The sub-clones of the BACs containing AFLP marker fragments were screened by hybridization to the SSR probes (CT)_{10}, (GT)_{10}, and (AGG)_{10}. The sub-clones identified as containing SSRs were end-sequenced. Eight SSRs were identified and primers were designed from the flanking sequences for each SSR (see Table 1).

SSR Screening of Three Populations Segregating for PPV Resistance

‘Vestar’ × ‘LE-3246’, ‘Lito’ × ‘Lito’, and ‘Goldrich’ × ‘Valenciano’ populations were screened with 8 different primer combinations for apricot SSR loci. A total of 6, 4, and 2 of the 8 SSRs were determined to be polymorphic in the ‘Vestar’ × ‘LE-3246’ population, ‘Goldrich’ × ‘Valenciano’, and ‘Lito’ × ‘Lito’ populations respectively.

DISCUSSION

In the past, AFLP markers have been used to develop genetic linkage maps for traits such as resistance to PPV (Salava et al., 2002a, 2002b; Vilanova et al., 2003), root-knot nematode resistance (Lu et al., 1998), and resistance to powdery mildew (Dirlewanger et al., 1996). The conversion of AFLP markers into SSR markers could prove to be a valuable tool for the plant breeder to use MAS (marker assisted selection) to screen for resistant cultivars in apricot, and due to their high transportability, SSR markers could potentially allow screening for resistance across species. In this study, 8 SSR markers were screened in three separate populations segregating for resistance to PPV, ‘Vestar’ × ‘LE-3246’, ‘Lito’ × ‘Lito’, and ‘Goldrich’ × ‘Valenciano’. A total of 6, 4, and 2 of the 8 SSRs were determined to be polymorphic in the ‘Vestar’ × ‘LE-3246’ population, ‘Goldrich’ × ‘Valenciano’, and ‘Lito’ × ‘Lito’ populations respectively. Further analysis of the segregation of these markers in the three populations and mapping of the SSRs in the respective populations is necessary to determine if they are linked to PPV resistance in all these populations.

ACKNOWLEDGEMENTS

Thanks to the people from Dr. Abbott’s Laboratory in Clemson University and the group from IVIA in Valencia, Spain for all of their help and guidance and to the
USDA/ARS cooperative agreement number 58-1920-1-132 for funding this research.

Literature Cited


Construction of a genetic linkage map and identification of AFLP markers for resistance to root-knot nematodes in peach rootstocks. Genome 41: 199-207.


### Tables

Table 1. Total number of sequences analyzed; number of clones containing the primary, secondary, and tertiary sequences for each AFLP fragment cloning experiment; number of confirmed positive BACs per AFLP probe and SSRs developed from each AFLP.

<table>
<thead>
<tr>
<th>AFLPs</th>
<th>No. of clones sequenced</th>
<th>No. of Clones/primary sequence</th>
<th>No. of Clones/secondary sequence</th>
<th>No. of Clones/tertiary sequence</th>
<th>No. of Apricot BAC Clones</th>
<th>SSR Markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>EAA/MCAG</td>
<td>6</td>
<td>5</td>
<td>1</td>
<td>0</td>
<td>4</td>
<td>aprigms17</td>
</tr>
<tr>
<td>EAG/MCATT</td>
<td>7</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>aprigms19</td>
</tr>
<tr>
<td>EAT/MCCT</td>
<td>5</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>aprigms20</td>
</tr>
<tr>
<td>ETC/MCCT</td>
<td>7</td>
<td>5</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>aprigms24</td>
</tr>
</tbody>
</table>