Development of a Sequence-tagged Site for the RAPD Marker Linked to Leaf Spot Resistance in Olive

Genet Teshome Mekuria, Margaret Sedgley, and Graham Collins

Department of Horticulture, Viticulture and Oenology, Adelaide University, Waite Campus, Glen Osmond, South Australia 5064, Australia

Shimon Lavee

Institute of Horticulture, The Volcani Center, Bet-Dagan, Israel

Abstract. A sequence-tagged site (STS) was developed to identify a genetic marker linked to resistance to olive leaf spot caused by the pathogen, Spilocea oleaginea (Cast) (syn. Cycloconium oleaginum) (Ogawa and English, 1991). The disease has been recorded in all Mediterranean olive-growing countries since the mid 1800s, and it has been recognized as the most important leaf disease of olives in China, California, South Africa, South America, and Australia (Hughes, 1953; Ogawa and English, 1991).

The pathogen attacks mainly leaves, producing lesions and circular spots on the upper surfaces, but in rare cases fruit and fruit stems may also be attacked (Shabi et al., 1994). The lesions first appear as small sooty blotches, and later develop into dark green to black circular spots, which, if numerous, result in leaf yellowing and abscission (Ogawa and English, 1991). Because most infected leaves fall prematurely, small branches weaken and subsequently die, resulting in a marked reduction in both flower bud differentiation and fruit set in subsequent years (Shabi et al., 1994). Hartmann (1957) reported that in California, some trees show up to 20% reduced yield due to partial defoliation caused by S. oleaginum. A complete loss of yield of some sensitive cultivars can occur under extreme, inductive, climatic conditions (Lavee et al., 1999).

To improve the utility of randomly amplified polymorphic DNA (RAPD) markers in breeding programs, Paran and Michelmore (1993) developed sequence characterized amplified regions (SCARs) by using longer and more specific primers developed from the sequences of RAPD fragments. These longer primers generate a sequence-tagged site (STS) as described by Olson et al. (1989). Such markers have been developed for disease resistance in many plant species, such as downy mildew (Bremia lactucae Regel) resistance in lettuce (Lactuca sativa L.) (Paran and Michelmore, 1993), and red stele root rot (Phytophthora fragariae Hickman var. fragariae) resistance in strawberry (Fragaria ×ananassa Duch.) (Haymes et al., 2000).

Using bulk segregant analysis (BSA), a RAPD marker linked to resistance to olive leaf spot disease was identified previously (Mekuria et al., 2001) using DNA from a segregating population obtained from a breeding program in Israel. The present investigation reports development of specific primers to generate an STS linked to leaf spot in olives based on that RAPD marker.

Materials and Methods

PLANT MATERIALS. Parents and progeny were selected from a breeding program at the Volcani Center, Bet-Dagan, Israel. This breeding program was aimed at developing olive genotypes resistant to S. oleaginua, and has been underway since the early 1980s. Four parents from the breeding program were disease-free over a period of 20 years and were designated resistant. The fifth parent showed occasional slight infection and was designated semiresistant as described in Mekuria et al. (2001). These trees were used as female parents in hybridizations with four commercial cultivars that showed different degrees of susceptibility to the disease including semiresistance (Mekuria et al., 2001). Progeny derived from these crosses were screened for olive leaf spot under field conditions for at least 8 years and designated as either resistant or susceptible.

DNA samples from the five female parents, the four male parents, and 34 of the progeny were used to identify a RAPD marker linked to leaf spot resistance in olives (Mekuria et al. 2001). In the present study the same DNA samples were used to develop an STS marker. In addition, DNA extracted from 12 commercial olive cultivars, ‘Koroneiki’, ‘Leccino’, ‘Corregiola’, ‘SA Verde’, ‘Sevillano’, ‘Ascolano’, ‘Kalamata’, ‘Picual’, ‘Mission’, ‘Novo’, ‘Barouni’, and ‘Attica’ were included in the analysis (Table 1).
Table 1. Field response of selected olive cultivars to olive leaf spot, and detection of STS marker.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Source of leaves</th>
<th>Identity of cultivar confirmed</th>
<th>Field response</th>
<th>STS marker band</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascolano</td>
<td>USA</td>
<td>Yes</td>
<td>NA</td>
<td>–</td>
</tr>
<tr>
<td>Attica</td>
<td>Australia</td>
<td>No</td>
<td>S</td>
<td>–</td>
</tr>
<tr>
<td>Barouni</td>
<td>Australia</td>
<td>No</td>
<td>S</td>
<td>–</td>
</tr>
<tr>
<td>Corregiola</td>
<td>Australia</td>
<td>Yes</td>
<td>R</td>
<td>+</td>
</tr>
<tr>
<td>Kalamata</td>
<td>Australia</td>
<td>Yes</td>
<td>NA</td>
<td>–</td>
</tr>
<tr>
<td>Koroniki</td>
<td>Greece</td>
<td>Yes</td>
<td>R</td>
<td>+</td>
</tr>
<tr>
<td>Leccino</td>
<td>Italy</td>
<td>Yes</td>
<td>R</td>
<td>+</td>
</tr>
<tr>
<td>Mission</td>
<td>USA</td>
<td>Yes</td>
<td>S</td>
<td>–</td>
</tr>
<tr>
<td>Novo</td>
<td>Israel</td>
<td>No</td>
<td>S</td>
<td>–</td>
</tr>
<tr>
<td>Picual</td>
<td>Israel</td>
<td>Yes</td>
<td>S</td>
<td>–</td>
</tr>
<tr>
<td>SA Verdale</td>
<td>Australia</td>
<td>Yes</td>
<td>NA</td>
<td>+</td>
</tr>
<tr>
<td>Sevillano</td>
<td>USA</td>
<td>No</td>
<td>NA</td>
<td>–</td>
</tr>
</tbody>
</table>

3Mekuria et al., 1999; J. Guerin, unpublished.
3FAO, 1996; J. Archer, person communication. R = resistant; S = susceptible; NA = information not available.
3(+)/Mark absent; (–) marker absent.

**Isolation and Purification of the RAPD Leaf Spot Marker.**
DNA was amplified using the primer OPA115'CAATCCGGCGT 3' as described in Mekuria et al. (2001), and the polymerase chain reaction (PCR) products were separated on 6% polyacrylamide (Accugel 40% 19:1 acrylamide: bisacrylamide, Kimberly Res., Atlanta). After silver staining the gel, the 780 base pair (bp) RAPD marker was excised from either fresh or dried polyacrylamide, and purified based on the method of Sambrook et al. (1989). Approximately 40 ng of the purified DNA was subjected to reamplification using the same protocol as for RAPD analysis, except that the final extension time was increased by an additional 5 min. The quality of the reamplified PCR product was assessed by separation on 1.5% agarose in Tris-borate-EDTA (TBE) buffer (Sambrook et al., 1989).

**Cloning and Sequencing the 780-bp Marker.** The reamplified PCR product was cloned into pCR2.1-TOPO and the plasmid was introduced into E.coli with the TOPOTA Cloning Kit (Invitrogen Corp., Carlsbad, Calif.) according to the manufacturer’s instructions. Plasmid DNA was purified from white colonies using a standard protocol involving alkaline extraction (Promega Corp., Madison, Wis.). Cloned DNA was analyzed by cleavage with EcoRI restriction enzyme (10 units/µL) (Boehringer, Mannheim, GmbH, Germany) and separated by electrophoresis on 1.5% agarose gels in TBE buffer.

Sequencing reactions were performed using the dye deoxy terminator sequencing kit of Applied Biosystems (Foster City, Calif.) with the M13 forward and reverse primers. Resulting sequencing reactions were analyzed on either an ABI 337A or ABI 3700 DNA analyzer (Applied Biosystems, Foster City, Calif.), and sequences were aligned with ClustalX (Thompson et al., 1997) and BioEdit Sequence Alignment Editor ver. 4.8.1 (N. C. State Univ., Raleigh, N.C.).

**Primer Design and Analysis.** Primers were designed either visually by adding 10 to 14 bases to the 3’ end of the sequence of the original 10-mer (Paran and Michelmore, 1993), by using the computer program PRIMER (version 0.5, Whitehead Inst. for Biomolecular Res., Cambridge, Mass.), or by NetPrimer (Primer Biosoft International, Palo Alto, Calif.). With NetPrimer, primers were designed initially by adding bases to the 3’ end of the sequence of the original 10-mer. However, in most cases, potential problems, such as primer dimer and hairpin sequences were indicated by NetPrimer. Therefore, the program was permitted to design primers based on internal sequences within the cloned DNA without regard to the sequence of the 10-mer (Williamson et al., 1994). Factors such as primer length, guanine-cytosine content, and melting temperature were taken into account to avoid possible primer dimer or secondary structure formation, to match melting temperatures, and to achieve appropriate internal stability.

Primer pairs were screened for their suitability to use as sequence specific primers using individuals from the olive population segregating for resistance to leaf spot disease. The reaction mixture for PCR was identical to that used for RAPD amplification (Mekuria et al., 2001) but the specific forward and reverse primers were used at 0.25 µM each, and the cycling conditions were altered to 35 cycles of 3 min at 95 °C, 45 s at X °C, 1 min at 72 °C, with a final extension of 10 min at 72 °C, where X is the optimal annealing temperature for each primer pair. The PCR products were separated on 3% agarose gels (Nusieve:Seakem LE agarose, 3:1) in TBE buffer, stained with ethidium bromide, and visualized under ultraviolet light.

**Results**

Purified plasmid DNA was digested with EcoRI, and the digested products were resolved by electrophoresis on agarose gels. A number of fragments in the expected size range of 780 bp were identified. One of these fragments, representing about 28% of recombinants, resolved into four bands, indicating the presence of three internal EcoRI sites. Because it was not possible to readily identify the resistance marker, 26 cloned fragments were sequenced. A total of nine unique inserts were identified, varying in size from 765 to 814 bases.

**STS Analysis.** The largest and the smallest cloned RAPD fragments were excluded from further analysis, and a total of 10 pairs of STS primers was selected from the remaining seven RAPD sequences identified. Three primer pairs were designed visually, three by PRIMER, and four by NetPrimer. Primer pairs designed visually amplified PCR products of the expected length, but none generated a polymorphism between the resistant and susceptible samples tested. Two of the primer pairs automatically selected by the PRIMER computer program, produced the expected size, but none produced the expected polymorphism.

Of the four primer pairs synthesized using NetPrimer, only one pair, named G7F and G7R, amplified a polymorphic band between the resistant and susceptible individuals. Other bands were also present (Fig. 1). The G7F and G7R primers were selected from the RAPD sequence of 773 bases produced from the cloned fragments that had three internal EcoRI restriction sites (Fig. 2). The sequences of the primers are G7F 5’-CATCACA CACTCCACTCTC-3’, located between bases 34–54, and G7R 5’-CAGCTACATCCAATCCCTTC-3’, located between bases 655 and 675. These primers were then PCR amplified to obtain the fragments used for further analysis. The product was therefore expected to be shorter than the original RAPD marker by 132 bp.

**Analysis of the STS Primer Pair G7F and G7R.** The primer pair G7F and G7R was tested on genomic DNA from nine parents and 34 progeny from the segregating population. The four resistant parents, and 11 of the 17 resistant progeny produced the STS marker. The marker was also present in three of the 17 progeny.
designated as susceptible, but not in any of the susceptible parents or the semiresistant parents. The primers were further tested using DNA from some commercial olive cultivars that are reported to show various levels of resistance to olive leaf spot (Table 1).

Presence or absence of the STS marker is shown by a representative selection of parents, progeny, and commercial cultivars in Fig. 1.

Two STS fragments, one from a resistant parent and the other from a resistant progeny, were cloned, and the sequences of five inserts were compared to that of the original RAPD marker. All five STS fragments showed an extra 10 bp between bases 264 to 273 (5′–3′) of the RAPD sequence. They showed 99.1% homology to each other, and 98% homology to the original RAPD band (excluding the extra 10 bp) (Fig. 2).

**Discussion**

The STS marker occurred in 65% of the resistant segregating progeny (field evaluated for 8 years), and 100% of the resistant parents (field evaluated for 20 years). When the presence of the STS marker was applied to all parents and progeny designated as resistant, the marker was present in 71.4% cases. On the other hand, 87% of the parents and progeny showing susceptibility to the disease did not produce the STS marker.

The primers were further used to test the DNA from 12 olive cultivars that showed various levels of olive leaf spot disease. Cultivars such as ‘Koroneiki’ and ‘Leccino’ that show resistance to olive leaf spot were distinguished from ‘Barouni’ and ‘Mission’, which are reported to be susceptible (FAO, 1996).

The results obtained using the STS primers were generally consistent with those obtained with the RAPD primer (Mekuria et al., 2001). Both the RAPD and the STS primers produced a marker with the four parents that were designated as resistant under field conditions, but not with the parents that were designated as either susceptible or semiresistant. Two field resistant progeny and two field susceptible progeny produced the RAPD marker but not the STS marker. On the other hand, one resistant progeny produced the STS marker but not the RAPD marker. Five progeny designated as field resistant produced no marker for resistance with either the RAPD or the STS primers, and so these progeny could be considered susceptible. One progeny, originally designated as field resistant but recently reviewed as susceptible (Mekuria et al., 2001), did not produce either the RAPD marker or the STS marker. This finding highlights the danger of designating progeny as resistant on the basis of relatively short-term field observation. Three progeny that were designated as field susceptible produced the marker band with both the RAPD and the STS primers. These progeny showed infection only after 8 years in the field. Possible reasons for this inconsistency could include involvement of a number of genes (not yet identified) that modify expression of resistance under field conditions, and the existence of more than one genotype of the causal organism. Individuals that show the marker band appear to inherit this trait only from parents showing complete resistance because four progeny derived from a cross between two semiresistant parents.

---

**Fig. 1.** STS marker (arrow) amplified with primers G7F and G7R and separated on a 3% agarose gel. Lane M = 100 bp ladder; lanes 1 and 13–15 = resistant parents; lanes 2–7 and 19–21 = resistant progeny; lanes 16–18 = susceptible parents; lanes 8–12 and 22–24 = susceptible progeny; lane 25 = ‘Koroneiki’ (resistant); lane 26 = ‘Leccino’ (resistant); lane 27 = ‘Attica’ (susceptible); and lane 28 = ‘Mission’ (susceptible).

**Fig. 2.** Sequence homology between the STS marker and the original RAPD sequence. The direction of the sequences were 5′ to 3′.
Brady, J.L., N.S. Scott, and M.R. Thomas. 1996. DNA typing of hops progeny resistant to olive leaf spot in breeding programs. (Jones et al., 1997) and will improve the efficiency of selecting for reproducible between laboratories than the RAPD 10-mer primer marker. The primers used to detect the STS marker are more sequence specific primers designed from the sequence of a RAPD linked to resistance to olive leaf spot was developed using resistant progeny at an early stage. In this study an STS marker makes it possible to select field observation alone takes many years. Development of a molecular marker linked to this trait makes it possible to select resistant progeny at an early stage. In this study an STS marker linked to resistance to olive leaf spot was developed using sequence specific primers designed from the sequence of a RAPD marker. The primers used to detect the STS marker are more reproducible between laboratories than the RAPD 10-mer primer (Jones et al., 1997) and will improve the efficiency of selecting for progeny resistant to olive leaf spot in breeding programs.

**Literature Cited**


