J. Amer. Soc. Hort. Sci. 127(4):673–676. 2002.

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

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Additional index words. STS, marker, *Spilocea oleaginea*, *Olea europaea*

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* Cast.). The STS was based on a randomly amplified polymorphic DNA (RAPD) marker of about 780 base pairs (bp) linked to olive leaf spot resistance. Several primer pairs were developed to flank the sequence, and one pair produced the expected polymorphism between resistant and susceptible individuals tested, and was used as an STS marker. This primer pair was tested against parents and 34 individuals from a population segregating for resistance to olive leaf spot, and 12 commercial olive (*Olea europaea* L.) cultivars showing various levels of resistance to the disease. The STS marker was present in 71.4% of the parents and progeny that were designated as resistant, and was absent in 87% of the parents and progeny showing susceptibility. These primers were also able to distinguish cultivars such as 'Koroneiki' and 'Leccino', that are reported to show resistance to olive leaf spot, from 'Barouni' and 'Mission', that are reported to be susceptible. This is the first report of a STS marker for olive, and its use will assist greatly in screening olive progeny for resistance to leaf spot in breeding programs.

Olive leaf spot, peacock spot, peacock eye, and bird's eye spot are synonyms for the same disease caused by the fungal pathogen, *Spilocea oleaginea* (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. oleaginea*, 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 Verdale', 'Sevillano', 'Ascolano', 'Kalamata', 'Picual', 'Mission', 'Novo', 'Barouni', and 'Attica' were included in the analysis (Table 1).

Received for publication 25 June 2001. Accepted for publication 21 Feb. 2002. G.T.M was supported by an Australian Post Graduate Award Scholarship. The research was supported by the Australian Research Council.
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Table 1. Field response of selected olive cultivars to olive leaf spot, and detection of STS marker.

Cultivar	Source of leaves	Identity of cultivar confirmed[z]	Field response[y]	STS marker band[x]
Ascolano	USA	Yes	NA	–
Attica	Australia	No	S	–
Barouni	Australia	No	S	–
Corregiola	Australia	Yes	R	+
Kalamata	Australia	Yes	NA	–
Koronieiki	Greece	Yes	R	+
Leccino	Italy	Yes	R	+
Mission	USA	Yes	S	–
Novo	Israel	No	S	–
Picual	Israel	Yes	S	–
SA Verdale	Australia	Yes	NA	+
Sevillano	USA	No	NA	–

[z]Mekuria et al., 1999; J. Guerin, unpublished.
[y]FAO, 1996; J. Archer, person communication. R = resistant; S = susceptible; NA = information not available.
[x](+)Marker present; (–)marker absent.

Isolation and purification of the RAPD leaf spot marker. DNA was amplified using the primer OPA11 5´CAATCGCCGT 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 pCR 2.1-TOPO and the plasmid was introduced into *E.coli* with the TOPO TA 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 *Eco*RI 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 *Eco*RI, 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 *Eco*RI 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 *Eco*RI restriction sites (Fig. 2). The sequences of the primers are G7F 5´-CATCAC-CACTCCACTCCTCTC-3´, located between bases 34–54, and G7R 5´-CAGCATCTCCATAATCCTTTC- 3´, located between bases 655 and 675. These primers were designed to amplify the sequence from base 34–675, and 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

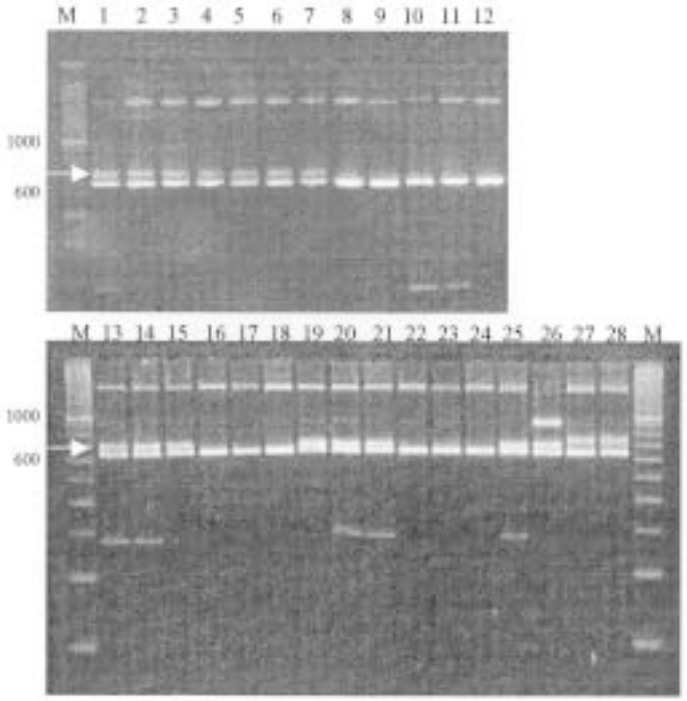

Fig. 1. STS marker (arrow) amplified with primers G7F and G7R and separated on a 3% agarose gel. Lane M = 100 bp ladder; lanes 1 and 13–15 = resistant parents; lanes 2–7 and 19–21 = resistant progeny; lanes 16–18 = susceptible parents; lanes 8–12 and 22–24 = susceptible progeny; lane 25 = 'Koronieki'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

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

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                                                    G7-F
RAPD  CAATCGCCGT CGGCCAGCAT TTCAATCAAA GACCATCACC ACTCCACTCC TCTCAACCAG GCACATCCAG TACCATCATG GGTCACCTGC AATTTTGTCC  100
STS                                   CATCACC ACTCCACTCC TCTCAACCAG GCACATCCAG TACCATCATG GGTCACCTGC AATTTTGTCC

RAPD  AGAATCGAGT GTGCTTGTGA AAGTAGACCG TGAGTTTTGA AATAGATTCG GCCCCAACAA AATTGATGAA ATTTTATTAA TAGGTAAGCA TTTCGTTTTT  200
STS     AATCGAGT GTGCTTGTGA AAGTTGACCG TGAGTTTTGA AATAGATTCG GCCCCAACAA AATTGATGAA ATTTTATTGA TAGGTAAGCA TTTCGTTTTT

RAPD  TATTTAATTC TTTATGGGTT AATTTTAACG ATTTGTGAAT TCACATTCTA ATACTTCGTC TTT          CCCCTAT CCCTGCAATC TCTCCTCTTA  290
STS   TATTTAATTC TTTATGGGTT AATTTTAACG ATTTGTGAAT TCACATTCTA TTACTTCGTC TTTCTACGTC TTTCCCCTAT CCCTGCAATT TCTCCTCTTA

RAPD  TGTTGTGGAA TTGGCAATGA GATTTTCAAA GAAATGTATG TCTCTT GTT CTTTATTGTT GAATTCCATG CTGCCGTTAT TGGAATTTTC TGCATTTTGT  389
STS   TGTTGTGGAA TTGGCAATGA GATTTTCAAA GACATGTATG TCTCTTTGTT CTTTATTGTT GAATTCCATG CTGCTGTTAT TGGAATTTTC TGCATTTTGT

RAPD  TTGTTCCTCT TGGTCGGATA GGTGGTGGGA CGTTGATATT CGGGAGACTT TTAGGAGTGT GATTAAGAGG CTTGATGACT GATGAGTATT TGAATTCGTG  489
STS   TTGTGCCTCT TGGTCGGATA GGTGGTGGGA CGTTGATATT CGGGAGACTT TGAGGAGTGT GATTAAGAGG CTTGATGACT GATGAGTATT TGAATTTGTG

RAPD  GGATCGGAGA CGGGAAAATG TTGGGAGGGC TGATGTGAAA AAGAGAGGTG AGGAGGAAAA TGAAGCGTGG AATTGGGAGC GGTGGAGGAA GCATTTTGCT  589
STS   GGATCGGAGA CGGGAAAATG TTGGGAGGGC TGATGTGAAA AAGAGAGGTG AGGAGGAAAA TGAAGAGTGG GATTGGGAGC GGTGGAGGAA GCATTTTGCT
                                                                                  G7-R
RAPD  GAGGTCGATA AGCAATAGCG TATTTATCTC GATTTTGAAG TCACAGTCGG CTTTTGCTAC TAATAGAAAG GATTATGGAG ATGCTGCTAG GCTCACGGTG  689
STS   GAGGCCGATA AGCAATAGCG TACTTATCTC GATTTTGAAG TCACAGTTGG CTTTTGCTAC TAATAGAAAG GATTATGGAG ATGCTG

RAPD  GCAATTGCAG CTGCAGCAAC AAAATGTTGG CAGAGTAATG TCTCATCTGA AGAAAGGTGT TCNGGAAGAA TATTACGGCG ATTG 773
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Fig. 2. Sequence homology between the STS marker and the original RAPD sequence. The direction of the sequences were 5′ to 3′.

(MAS-5 x 'Manzanillo') did not produce either the RAPD or the STS marker.

Nine different sequences, varying from 765 to 814 bp, were identified from the reamplified PCR products originating from the marker band produced by OPA 11. It was assumed from resolution of the bands after electrophoresis that five sequences from 773 to 779 bp were comigrating as a single RAPD band, and that others outside this range were produced as artifacts during the reamplification process. This finding confirms those of Brady et al. (1996) and Hausner et al. (1999) that a single RAPD band may consist of several similar-sized DNA fragments each with a different sequence. In addition to the marker for olive leaf spot resistance, other products were produced during PCR with the primer pair G7F and G7R. Amplification of multiple PCR products from sequence specific primers is reported in other studies (Brady et al., 1996; Lu et al., 2000; Olsen and Eckstein, 1989).

Screening progeny for resistance to olive leaf spot based on 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.

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