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Development of SCAR markers in olive (*Olea europaea*) by direct sequencing of RAPD products: applications in olive germplasm evaluation and mapping

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Abstract RAPD markers generated by mixtures of two different 10-mer primers were developed for eight different olive cultivars used as parental lines in olive-breeding programs. Two RAPD bands were converted into dominant SCAR markers by direct sequencing of the RAPD products, avoiding the costly and time-consuming cloning step. The SCARs generated have maintained the original RAPD polymorphism among the cultivars and segregated according to Mendelian inheritance. Preliminary results suggest the use of the SCAR SCOeMS-2 for the marker-assisted selection of the high flesh/stone ratio. This strategy provides a rapid method for the characterization of RAPD markers and for the development of PCR-based markers with applications in olive mapping, paternal testing and germplasm characterization. The use of these markers in multiplexed PCRs, and the direct ethidium bromide detection of the PCR products in the test tube, facilitate their efficient and reliable breeding applications.

Keywords Marker-assisted selection · Molecular breeding · *Olea europaea* · RAPD-PCR · Cultivar identification

Introduction

Olive breeding has been mostly based on the local empiric selection carried out by growers (Rallo 1994) and has been determined by the traditional vegetative reproduction system. Efforts for the development of new varieties by systematic breeding have produced very little results (Lavee 1978; Lavee et al. 1986), mainly because it can only be approached in the long term.

The main reason for the scarcity of breeding efforts leading to the development of new varieties is the length of the juvenile period. Molecular markers have been proposed as good early selection techniques (marker-assisted selection, MAS) by their association with agronomic traits in trees (Janick and Moore 1996). Olive cultivar identification is a subject of great economic importance, mainly in periods (like now) of expansion in its cultivation. For such periods large quantities of the more demanding varieties are requested. Additionally, due to the length of the non-productive period, non-detected misleads in variety identification may increase considerably the establishment of plantation costs. Therefore, there is an urgent need for the development of early identification techniques. The identification of olive tree cultivars has been traditionally carried out by morphological and agronomic traits. However, they are dependent on environmental or cultivation factors. Identification based on the analysis of gene products, such as isozymes, has also been used (Trujillo et al. 1995). Nevertheless, it is still limited by the relatively small number of polymorphisms detected, and by the possibility that isozyme expression can also be altered by environmental conditions.

Nowadays, the practical use of genetic markers is predominantly performed through PCR (polymerase chain reaction)-based techniques. Standard PCR is conditioned by a priori knowledge of the nucleotide sequences flanking the locus to be amplified. As there is little or no sequence information of the olive genome available, random amplified polymorphic DNA (RAPD; Welsh and McClelland 1990; Williams et al. 1990) is a simple and reliable source of polymorphism that has already been used for olive cultivar identification and phylogenetic studies (Fabbri et al. 1995, Wiessman et al. 1998, Belaj et al. 2000). The conversion of such markers in sequence-characterized amplified regions (SCARs; Paran and Michelmore, 1993) by

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the development of longer (i.e. more specific) primers from the RAPD sequences, significantly improves the reproducibility and reliability of PCR assays, and therefore their utility for marker-assisted breeding. Nevertheless, a such procedure includes the cost and time-consuming step of cloning PCR products. Hernández et al. 1999 reported the development of SCAR markers in the wild barley *Hordeum chilense*, using a direct sequencing approach and thus avoiding the cloning step. This simplified strategy for the generation of SCARs, in addition to their possible use as allele specific amplified polymorphisms (ASAPs; Gu et al. 1995) or in multiplexed PCRs, makes this type of molecular marker particularly suitable for marker-assisted breeding programs. We have used this technology to develop SCAR markers useful for olive cultivar identification and mapping.

Materials and methods

Plant material

Eight *Olea europaea* cultivars from the Germplasm Bank of Córdoba, Spain, were studied: 'Picual', 'Manzanilla de Sevilla', 'Lechín de Sevilla', 'Gordal Sevillana', 'Domat', 'Arbequina', 'Frantoio' and 'Hojiblanca'.

DNA extraction

DNA was extracted from young leaf tissue using the CTAB method (Murray and Thompson 1980) with some modifications. Tissue (0.7–1 g) was ground in liquid N_2. Before the tissue thawed, 3 ml of CTAB buffer containing 0.5% Na bisulphite and 25 mM of DTT were added. The solution was incubated 40 min at 65°C with occasional mixing. After the tissue was extracted once with the same volume of 24:1 chloroform/octanol, the aqueous phase was isopropanol-precipitated and spooled out using a glass hook, washed in 70% ethanol and air-dried. The DNA was dissolved in about 500 µl of TE buffer. RNase was added (0.03 mg) and the DNA was dissolved overnight at 4°C. The obtained DNA stock solution was diluted 1:20 in water for PCR amplification.

DNA amplification

Approximately 20–40 ng of genomic DNA were used in the amplification reactions, following the protocol described by Hernández et al. (1996). Random 10-mer primers were purchased from Operon Technologies (Alameda, Calif., USA) and the *AmpliTaq* DNA Polymerase Stoffel Fragment from PE Biosystems (Foster City, Calif., USA). Amplification was performed in a System 9600 cycler from the same manufacturer. The amplified products were resolved by electrophoresis on gels consisting of 1% (w/v) SeaKem agarose: 1% (w/v) NuSieve agarose from FMC (Rockland, Me., USA), and TBE buffer. Gels were stained with 0.5 µg of ethidium bromide/ml, and photographed with a GDS 5000 system CCD camera from UVP (Cambridge, UK).

Direct sequencing of RAPD products

The RAPD bands were excised from agarose gels and soaked in 20 µl of sterile-distilled milliQ water. As direct sequencing of the RAPD products is only possible if they are flanked by the two different oligonucleotides, the RAPD product origin was checked before sequencing. Verification of the RAPD product origin, band DNA purification and sequencing was performed following Hernández et al. (1999).

Designing SCAR primers and subsequent specific-amplification of genomic regions

Based on the sequences of the cloned RAPD products, pairs of oligonucleotide primers were designed using the programs Oligo 5.0 for MacOS from Molecular Biology Insights (Plymouth, Minn., USA), and PrimerSelect 3.03 for MacOS from DNAStar (Madison, Wis., USA) for specific amplification of the loci identified by RAPD markers. Primers were synthesized by Operon Technologies. Primer sequences are available from the first author.

PCR

Amplification reactions were carried out in 20-µl solutions containing 20–40 ng of DNA, 0.5 units of *AmpliTaq* Gold DNA Polymerase from PE Biosystems, 100 µM of each dNTP from Roche (Basel, Switzerland), 0.16 µM of each primer, 2.5 mM $MgCl_2$, 50 mM KCl and 10 mM Tris-HCl, pH 8.3. Amplification was carried out under the following conditions: 94°C for 10 min; 35 amplification cycles (15-s denaturation at 94°C, 20-s annealing at 69°C, 1 min of extension at 72°C); and a final extension of 6 min at 72°C. Amplified products were resolved by electrophoresis in 2% (w/v) agarose gels as previously described.

Direct detection of amplification products in the test tube

The same conditions of PCR as those used for the SCAR marker were used for direct detection of the amplification products by fluorescence in the PCR tubes, with the addition of 0.5 µg/ml of ethidium bromide (Higuchi et al. 1992). The amplification tubes were analyzed directly by ethidium bromide staining under UV light with the CCD camera, as previously described.

Multiplexed PCR

Amplifications were carried out in 20-µl reaction mixtures containing the same components used for single analysis, except that each of the primers was included at a concentration of 1 mM.

Results and discussion

The objective of this investigation was to identify suitable RAPD polymorphisms across olive cultivars and to transform them into more-specific SCAR markers in an efficient and reliable manner. Ten pairwise combinations of RAPD primers were screened for suitable polymorphisms. As an example, amplification patterns obtained using the 10-mer primer pairwise combination OPJ5 + OPJ6 is shown in Fig. 1. RAPD-PCR is a widely used technique in plant genome analysis, but the practical applications of such markers are enhanced by sequencing the RAPD products and converting them into more-specific assays (Paran and Michelmore 1993). Moreover, such conversion is more cost effective if the direct sequencing methodology (Hernández et al. 1999) is applicable. To test the use of such an approach for olive cultivar identification, mapping and paternity testing, two polymorphic bands obtained with the combination OPJ-05 + OPJ-06 were chosen for the development of SCAR markers. They were directly sequenced (GenBank accession numbers AF130731 and AF130733), thus avoiding the cloning step. The original RAPD markers

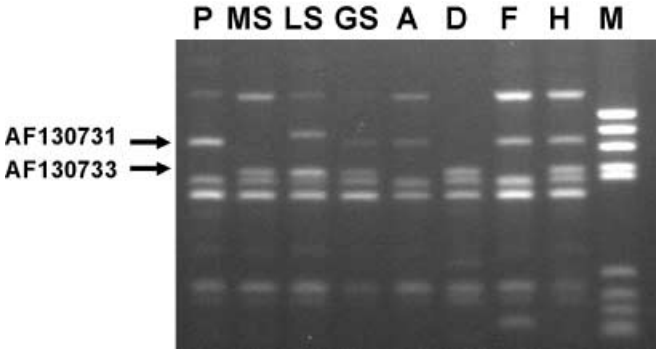

Fig. 1 RAPD amplification profiles of eight olive cultivars using the pairwise combination of primers OPJ-5 + OPJ-6. The RAPD markers corresponding to GenBank acc. nos. AF130731 and AF130733 are indicated by *arrows. Lanes* are as follows: *P* cv 'Picual'; *MS* cv 'Manzanilla de Sevilla'; *LS* cv 'Lechín de Sevilla'; *GS* cv 'Gordal Sevillana'; *A* cv 'Arbequina'; *D* cv 'Domat'; *F* cv 'Frantoio'; *H* cv 'Hojiblanca', *M* = molecular-weight marker

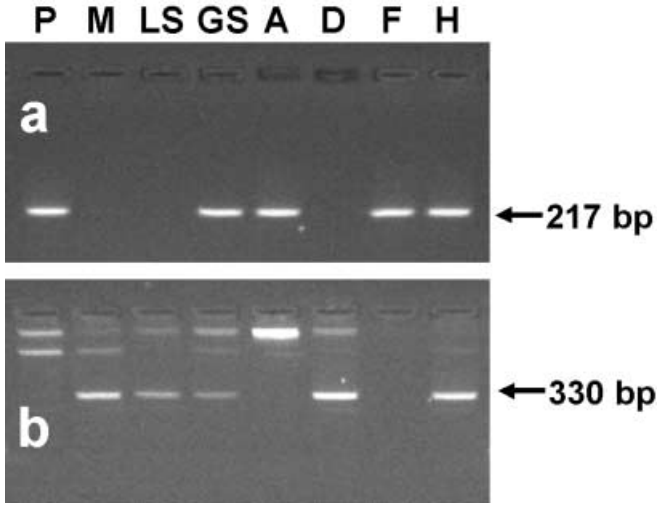

Fig. 2 Olive cultivar identification using SCARs SCOeP-1 **(a)** and SCOeMS-2 **(b)**. *Lanes* are as follows: *P* cv 'Picual'; *M* cv 'Manzanilla de Sevilla'; *LS* cv 'Lechín de Sevilla', *GS* cv 'Gordal Sevillana'; *A* cv 'Arbequina'; *D* cv 'Domat'; *F* cv 'Frantoio'; *H* cv 'Hojiblanca'

were successfully transformed into more-specific dominant SCAR assays (SCOeP-1 and SCOeMS-2).

The DNA amplification of eight olive cultivars using both dominant SCARs is shown in Fig. 2. Both show polymorphisms suitable for cultivar identification and resemble the original RAPD polymorphism. Both SCAR primer pairs were designed internally to the RAPD sequences, indicating that the original RAPD polymorphisms did not rely on the RAPD priming sites. Such a possibility of internal primer design increases the utility of the developed markers, allowing specific and robust multiplexing. SCAR SCOeP-1 amplifies a single band (Fig. 2a) and is thus suitable for a plus-minus screening by direct ethidium bromide detection in the amplification tubes (see Fig. 3b). Mendelian 3:1 genetic segregation is illustrated in Fig. 3 for the progeny of the selfing of cv 'Arbequina'.

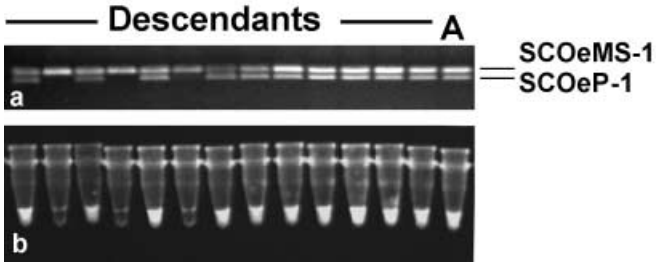

Fig. 3a, b Genetic segregation from SCAR SCOeP-1 in the descendants of the selfing of cv 'Arbequina'. **a** Multiplexed PCR consisting in the co-amplification of the SCAR SCOeMS-1 (407-bp fragment) as an internal amplification control and SCAR SCOeP-1 (217-bp fragment). **b** Single amplification of the SCAR SCOeMS-1 detected by direct ethidium bromide staining in the test tube

Table 1 Flesh/stone ratio and amplification of the SCAR marker SCOeMS-2

Cultivar	Amplification of SCOeMS-2	Flesh/stone ratio
'Arbequina'	–	4.6[a]
'Frantoio'	–	5.1[b]
'Picual'	–	5.6[a]
'Domat'	+	6.2[c]
'Lechín de Sevilla'	+	7.2[a]
'Gordal Sevillana'	+	7.3[a]
'Hojiblanca'	+	7.9[a]
'Manzanilla'	+	8.2[a]

[a] Barranco (1998)
[b] Del Río and Caballero (1994)
[c] R. de la Rosa, personal communication

The SCAR SCOeP-1 has an additional use in breeding, because it is amplified by the cultivars 'Picual' and 'Frantoio', used as pollen donors in the breeding program. For this reason, it has an application for paternity testing of F_1s when either of these two varieties is used as the male parent on crosses between homozygous males and females lacking this amplification product. Direct sequencing of RAPD products will therefore help to overcome the problem of the discrimination of selfing when crosses are used in olive breeding.

The RAPD marker AF130733 was initially chosen for SCAR development, because it was present in the Spanish table cultivars 'Manzanilla de Sevilla' and 'Gordal Sevillana', while absent in the oil cultivars 'Picual' and 'Arbequina'. In a first approach, its amplification suggested a putative association with a high flesh/stone ratio. The associated SCAR SCOeMS-2 maintained this RAPD polymorphism. With additional cultivars (like 'Domat', 'Lechín de Sevilla, 'Hojiblanca' and 'Frantoio') the tendency was well maintained both by the RAPDs (Fig. 1) and the SCARs (Fig. 2). The amplification of the SCAR SCOeMS-2 is associated with higher flesh/stone ratios, while the cultivars with lower flesh/stone ratios failed to amplify this marker, as shown in Table 1. Flesh/stone ratio is considered a very important commercial factor (Barranco 1998) since it controls

the fraction of the crop that produces oil (the flesh). This SCAR has therefore a putative use in germplasm evaluation and marker-assisted selection, but its discrimination power for flesh/stone ratio needs to be confirmed by the screening of a larger collection of cultivars.

The RAPD band AF130732 was cloned and sequenced (Hernández et al. in preparation) originating the SCAR SCOeMS-1. The unspecific SCAR SCOeMS-1 is amplified by every olive cultivar we have tested. Two primer pairs have been designed on the basis of its sequence, yielding PCR products of 146 and 407 bp respectively, providing internal amplification controls covering the range of PCR molecular weights for multiplexing (see Fig. 3 for an example).

In summary, the proposed methodology represents a simple and reliable approach for the generation of effective and specific molecular markers to assist in olive paternity testing, variety identification and mapping.

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