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Molecular analysis of RAPD DNA based markers: Their potential use for the detection of genetic variability in jojoba (*Simmondsia chinensis* L Schneider)

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Summary — We have applied the recently developed technique of random amplified polymorphic DNA (RAPD) for the discrimination between two jojoba clones at the genomic level. Among a set of 30 primers tested, a simple reproducible pattern with three distinct fragments for clone D and two distinct fragments for clone E was obtained with primer OPB08. Since RAPD products are the results of arbitrarily priming events and because a given primer can amplify a number of non-homologous sequences, we wondered whether or not RAPD bands, even those of similar size, were derived from different loci in the two clones. To answer this question, two complementary approaches were used: i) cloning and sequencing of the amplification products from clone E; and ii) complementary Southern analysis of RAPD gels using cloned or amplified fragments (directly recovered from agarose gels) as RFLP probes. The data reported here show that the RAPD reaction generates multiple amplified fragments. Some fragments, although resolved as a single band on agarose gels, contain different DNA species of the same size. Furthermore, it appears that the cloned RAPD products of known sequence that do not target repetitive DNA can be used as hybridization probes in RFLP to detect a polymorphism among individuals.

RAPD / RFLP / molecular markers / Jojoba

Introduction

A novel strategy for DNA fingerprinting that uses an arbitrarily short primer, generally of ten bases, for the low stringency amplification of multiple fragments from genomic DNA, was initially developed by both Williams *et al* [17] and Welsh and McClelland [15] and respectively termed randomly amplified polymorphic DNA (RAPD) and arbitrarily primed PCR (AP-PCR). Since this strategy requires no previous sequence information for fingerprinting and because it is fast, technically easy and requires a very small amount of genomic DNA to detect extensive polymorphisms among individuals, or groups of individuals, it has been greeted with particular enthusiasm. The fingerprints produced have been widely used to identify genotypes, varieties, cultivars and to determine the degree of variability in populations of wild and

cultivated plants and plants regenerated from *in vitro* culture.

RAPD clearly represents a useful method to identify a genetic polymorphism, however, despite the obvious advantages mentioned above, numerous questions have been raised by several authors and the molecular nature of the polymorphisms detected is generally unknown. The question of whether or not RAPD markers are reproducible across a variety of protocols and laboratory conditions has been addressed by Tinker *et al* [14]. Many parameters of the amplification reaction can influence the RAPD amplified DNA pattern and, just as important as the primer sequence, adequate resolution of amplification fragments is necessary to reveal the true number of amplified sites and to ensure that DNA patterns are reproducible [2]. As pointed out by Ellsworth *et al* [7], the appearance of artifactual variations represents a potential problem which may lead to an overestimation of the level of variation if artefacts are not clearly distinguished from true polymorphisms. Moreover, as pointed out by Muralidharan and Wakefield [11], the kinetics and characteristics of the reaction are complex

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In this paper, we report the generation of RAPD markers that allow a clear-cut discrimination between two clones of jojoba (*Simmondsia chinensis* L. Schneider), a crop of high economic interest which is grown for the exceptional quality of its seed oil. In order to gain information regarding differences in the type of DNA sequence represented by RAPD loci, some of the amplification products were cloned and sequenced and the question of degree of similarity between sequences contained within single bands resolved on agarose gels was addressed. Finally, we evaluated RAPD as a source of conventional RFLP probes following cloning of amplified fragments.

Materials and methods

Plant materials and tissue culture

Leaves from two clones originating from a Californian plantation, called clones D and E, were harvested from plants regenerated from tissue culture and grown in the greenhouse.

DNA isolation

Total cellular DNA was isolated from young leaves and assayed as reported by Corniquel and Mercier [3].

DNA amplifications, cloning and sequencing RAPD products

DNA amplification was performed as previously described by Corniquel and Mercier [3]. PCR products were ligated into the pCR vector (Invitrogen Corporation) and used to transform competent *E. coli* (INVαF) cells according to the TA cloning instruction manual. Bacteria were plated on LB agar plates containing ampicillin (50 µg/ml) and spread with 25 µl X-Gal (40 mg/ml). 18 white colonies were selected and screened for the presence of an insert of the correct size. Plasmid DNA was extracted and purified according to Qiagen protocols (Qiagen GmbH, Hilden, Germany). Double-strand DNA sequencing was performed using the Sequenase version 2.0 DNA sequencing kit (United States Biochemical).

Isolation of cloned probes and RAPD fragments

Cloned RAPD bands were separated from the pCR vector by digestion with *EcoRI* electrophoresed on 1% agarose gels, recovered by electroelution as described by Lebrun *et al.* [9] and used as hybridization probes. Alternatively, RAPD bands directly recovered from the gel by electroelution RAPD bands as above [9], were used as probes.

Restriction enzyme digestion, electrophoresis, blotting and hybridization

Genomic DNA samples (3 µg) were digested overnight with restriction enzymes according to the manufacturers' recommendations, in the presence of 2 mM spermidine. Restriction fragments were separated by electrophoresis on 0.8% agarose gels at 40 V for approximately 23 h in TAE buffer pH 8.3 [13]. After denaturation in 1.5 M NaOH/1.5 M NaCl (30 min) and neutralization in 1 M ammonium acetate/0.04 M NaOH

(30 min), DNA was transferred to Hybond N+ (Amersham) membranes by capillary action, using 1 M ammonium acetate/0.04 M NaOH. Probe labelling and hybridization were as previously described [3].

Results

Primer selection

A total of 30 primers were tested with DNA samples extracted from different individuals, in each of the two clones D and E. As seen in figure 1, a rather simple banding pattern showing polymorphic bands between the two jojoba clones was obtained with primer OPB 08. Whereas the 765 bp band is present in both clones, several bands were specifically amplified: bands at 960 bp and at 1150 bp in clone D and a 523 bp band in clone E. Individual reactions often yielded different relative amounts of the various amplified products as attested by differences in band brightness, except for the 1150 bp band in clone D. A band of lower intensity at approximately 3 kbp, common to both clones, was also seen. However, this weak band was not reproducibly observed in repeated runs.

Cloning and sequencing RAPD products of clone E

For each of the two cloned fragments of 523 bp and 765 bp, respectively called E₁ and E₂, one clone was used for plasmid preparation and sequencing. The nucleotide sequences of 523 bp and 765 bp confirmed on both strands have been registered in the EMBL/GenBank/DDBJ databases with accession numbers X82237/SCRAPD1 and X82238/SCRAPD2 respectively. These sequences which correspond to non-coding regions are rich in A and T (75% for E₁ and 64% for E₂), with stretches of 12 and 15 T respectively for E₁ and a stretch of 11 T for E₂. In addition, groups of trinucleotides such as (AAA), (TTT) in both sequences and (CCC) and (GGG) in E₁ were repeatedly found. However, these stretches and groups of nucleotides were not ordered in tandem repeats as in minisatellites or microsatellites. No similarity was found between the two sequences with the use of the homology comparison function of the sequence analysis program Microgenie (Beckman). The lack of similarity between the two sequences was further confirmed by the absence of cross-hybridization between the two cloned fragments, clearly indicating that these two fragments were generated from two different RAPD loci.

Southern analysis

In order to see if RAPD fragments of related sequences were amplified from clones D and E, Sou-

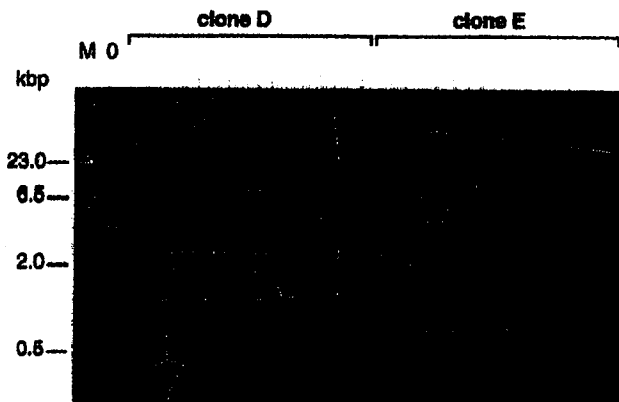


Fig 1. Ethidium bromide stained electrophoresis pattern of RAPD amplified joboba DNA. 25 ng of total genomic DNA from leaves of two clones D and E (nine individuals each) was amplified with primer OPB08, and separated on 1% agarose gel. Molecular size markers (λ DNA digested with *Hind*III) are in lane M. Lane O is a negative control containing the primer but no template DNA.

them transfers of the amplified products were sequentially probed with cloned and sequenced fragments E_1 and E_2 (fig 2A, B) and their corresponding amplification products e_1 and e_2 excised from agarose gels (fig 2C, D). Figure 2A shows that the cloned E_1 fragment does not hybridize with the amplified products from clone D. However, it hybridizes strongly with the 523 bp band e_1 and weakly with the 765 bp band e_2 . The fact that no homology was found between cloned fragments E_1 and E_2 strongly suggests that the amplification products resolved as a single band on agarose gels very likely contain different DNA species of similar size. From this hybridization analysis, we may expect that in the amplification products migrating as a single band of 765 bp, there is at least one fragment having a common origin with the cloned and sequenced fragment E_1 of 523 bp. Figure 2B shows that the cloned fragment E_2 not only hybridizes with the amplified products e_2 but also with a band of the same size and intensity in clone D (band d_1). This suggests that these are not fortuitous bands of similar size but that the same locus was, in fact, amplified in the two clones. The high degree of similarity between the amplified products e_2 and d_1 was also confirmed by using the fragment e_2 excised from the gels as a probe as shown in figure 2D. The same experiment was repeated with the 523 bp band excised from the gel as a probe. The pattern obtained was unexpected since the major band corresponded to band e_2 (fig 2C). Moreover, a signal of similar intensity consistent with the similarity previously shown to exist between e_2 and d_1 , was obtained with DNA samples from clone D. At least two reasons can be put forward to explain

the discrepancy between hybridization patterns obtained with the cloned and sequenced E_1 fragment (fig 2A) and the corresponding 523 bp band excised from agarose gels (fig 2C) when used to probe Southern transfers of the amplification products: i) the weak hybridization signal obtained with the 523 bp fragment excised from the gel as a probe may be related to the fact that there are significant differences in the brightness of this band among individuals, as seen in figure 1. The pattern seen in figure 2C might therefore be due to the loading of a low amount of amplification product e_1 as a result of a low amplification efficiency of the target DNA sequence; ii) the strongest signal obtained with the 765 bp fragment (fig 2C) is consistent with the fact that, as already mentioned, different DNA species are contained within single bands resolved on agarose gels. It is thus possible that one of the amplification products migrating in the band e_1 was derived from the same locus as E_2 and d_1 , with one of the E_1 priming sites located within the target sequence E_2 . If this is true (*ie* amplification products migrating as single bands of 523 bp and of 765 bp contain related sequences), the fact that no hybridization signal is observed with band e_1 , when the 765 bp fragment either cloned (E_2 , fig 2B), or excised from agarose gels (e_2 , fig 2D) is used as a probe, remains to be explained. The difference observed in figure 2 between panels A and B and between panels C and D respectively, most probably reflects differences in the kinetics of the hybridization reaction, with different C_0t values, whether the 765 bp fragment is the target (*ie* immobilized on the membrane after Southern transfer, in the μ g range), or the

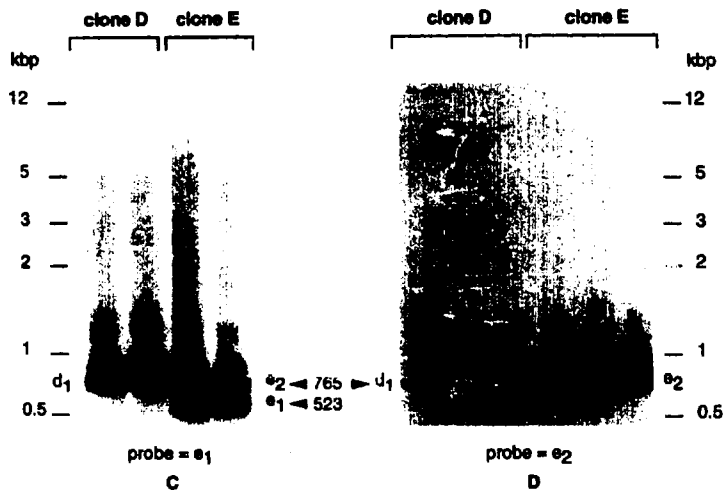
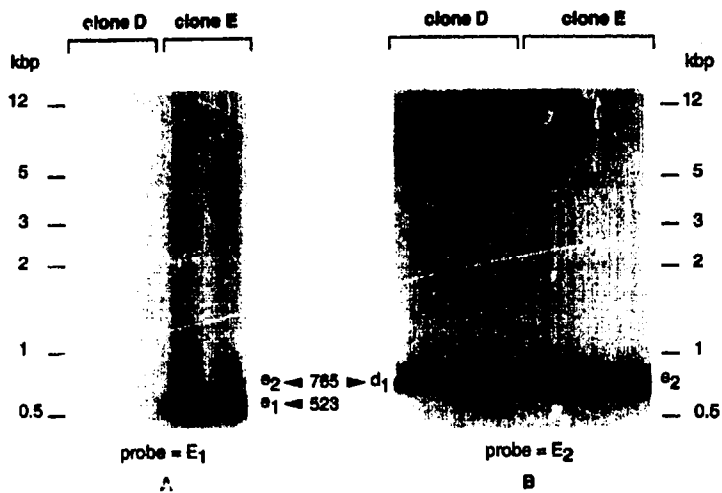


Fig 2. RAPD amplification products were separated on 1% agarose gel. The amplified fragments from this gel were transferred to a Hybond N⁺ membrane by capillary blotting, and hybridized with the cloned and sequenced 523 bp fragment E₁ (A), with the cloned and sequenced 765 bp fragment E₂ (B), with the amplified band of 523 bp (C) or with the amplified band of 765 bp (D) recovered from the gel by electroelution and radiolabelled with α -³²P dCTP. The 1-kbp ladder (Gibco-BRL) was used as size marker.

probe (*ie* in solution, with an amount in the ng range). This result is reminiscent of the data reported by Paran and Michelmore [12], who showed that, in lettuce, no detectable hybridization was obtained when total labelled genomic DNA was used to probe the amplification products, while bands were detected when the amplification products were used as a probe on genomic Southern. In addition, to support this statement we showed that the amplification product e₂ contains a very low amount of sequences related to E₁. In our cloning experiments (data not shown), among the colonies hybridizing with the amplification product e₂, 98% of recombinant colonies were also positive with the cloned and sequenced E₂ fragment.

After labelling, the cloned E₁ and E₂ fragments were also used to probe Southern transfers of genomic DNA digested by restriction enzymes. In addition to the polymorphism observed between the two clones with both probes, differences among individuals were also observed (data not shown). We should also note here that while no fragment equivalent to E₁ was amplified in clone D, the target sequence is indeed present in this clone as evidenced by the strong hybridization signal obtained with this cloned fragment as a probe (data not shown). This clearly indicates that cloned RAPD products can be used as an advantageous source of conventional RFLP probes.

Discussion

The discrimination between two clones of *jojoba* at the genomic level was achieved by RAPD with the use of a commercially available primer. Primer OPB08 was chosen because it gives easily detectable and well resolved bands. While the banding pattern was reproducible over repeated runs, with sufficient intensity to determine the presence or absence of RAPD fragments, variations between DNA samples were seen in the relative intensity of some bands. The amplification product E₁ exhibited differences in band brightness between individuals. The presence of bands of varying intensity in RAPD profiles has already been reported [2, 14]. Whether such results reflect differences in the availability of binding sites on genomic DNA and/or their relative affinities, or differences in the nucleotide sequence of the binding sites cannot be answered. In their pioneering work, Williams *et al* [17] have postulated that in the RAPD reaction, the composition of the amplification products is determined by competition between potential

priming sites in the template. Thus, the presence of bands of varying intensity could be related to alternative priming sites, some perfectly matched and others that include a degree of mismatching. In their study of primer-template interactions during DNA amplification fingerprinting with single arbitrary nucleotides, Caetano-Anollés *et al* [1, 2] proposed a model in which a single primer preferentially amplifies certain products due to competition for annealing sites between primer and hairpin loop structures of the template. In this paper, we have shown that a target sequence present in both clones, as evidenced by Southern analysis of genomic DNA with the cloned and sequenced fragment E₁, was only amplified in clone E. Such observations have also been made by Heun and Helentjaris [8] who reported that the presence of some amplification fragments can interfere with the amplification of others and that the absence of the latter fragments from some genotypes cannot assure the absence of their sequence. This confirms that not all amplified sites are equally well amplified and that some amplicons are preferentially amplified, as was pointed out by Caetano-Anollés *et al* [1].

As previously suggested by Dweikat *et al* [6], who used denaturing gradient gel electrophoresis for detection of DNA polymorphism generated by RAPD, our results show that some of the RAPD amplification products, migrating as single bands in an agarose gel, may contain contaminating sequences of the same size. It is therefore obvious that relying too heavily upon the amplification profiles after ethidium bromide staining is somewhat hazardous. That the use of RAPD to define fingerprints of individual genotypes should be viewed with some caution has also been recommended by Heun and Helentjaris [8]. Moreover, Tinker *et al* [14] found, using Southern analysis which is more sensitive to the presence of weak amplified products than ethidium bromide staining, that the number of scorable RAPD markers per primer was increased. While this relatively new and apparently simple technique has provided a means for easy and rapid genetic determination, the kinetics and characteristics of RAPD PCR are in fact complex. Not only does it require improved PCR conditions, but adequate resolution of amplification fragments is necessary to reveal the true number of amplified sites and to ensure the generation of patterns that are reproducible across a variety of protocols and laboratory conditions. Further analysis is then required to unequivocally ascertain the identity of samples with similar banding patterns.

While the extra effort of cloning and sequencing RAPD products is tedious, Southern analysis of amplification products and the use of cloned RAPD markers as hybridization probes may be necessary for critical purposes in order to ensure the relatedness among individuals within species (germplasm analysis). To that point, the use of the cloned E₁ fragment as an RFLP probe is very interesting since an extensive polymorphism was obtained among individuals in a population clonally propagated by *in vitro* tissue culture. The polymorphic RFLP pattern obtained between individuals in both clones strongly suggests that it targets a region of the genome which is highly variable.

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