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Characteristics of random amplified polymorphic DNA markers discriminating *Helianthus annuus* inbred lines

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Summary — Random amplified polymorphic DNA (RAPD) markers were used to discriminate between inbred lines of the sunflower *Helianthus annuus*. By testing one hundred 10-mer primers with DNAs from 4 lines, we obtained 40 primers showing polymorphism in at least one line. After selection of primers giving reproducible signals, 14 RAPD markers allowing the characterization of inbred lines of sunflower were obtained. Molecular studies of amplified fragments show that these markers are of the "presence/absence" type: a locus is characterized by 1 band for a line and by the lack of band for the other lines. Southern experiments with polymorphic RAPD fragments as probes gave smears as hybridization signals, indicating that the amplified fragments probably include highly repeated sequences.

***Helianthus annuus* = sunflower / genetic markers / RAPD / RFLP**

Résumé — Caractéristiques des marqueurs RAPD permettant la discrimination de lignées d'*Helianthus annuus*. Nous avons testé l'utilisation de marqueurs RAPD (random amplified polymorphic DNA) pour permettre la discrimination de lignées de tournesol cultivé *Helianthus annuus*. En testant 100 amorces décamères sur les ADN de 4 lignées, nous obtenons 40 amorces révélant un polymorphisme. Nous montrons qu'en sélectionnant les amorces donnant des signaux reproductibles on obtient 14 marqueurs RAPD permettant de caractériser des lignées de tournesol. L'étude moléculaire des fragments amplifiés montre que ces marqueurs sont de type «présence/absence» : un locus est caractérisé par une bande chez une lignée et l'absence de bande chez les autres lignées. Des expériences de Southern utilisant des fragments RAPD polymorphes comme sondes produisent des signaux d'hybridation en trainée, indiquant que les fragments amplifiés renferment vraisemblablement des séquences hautement répétées.

***Helianthus annuus* = tournesol / marqueurs génétiques / RAPD / RFLP**

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INTRODUCTION

Molecular markers have proved to be an attractive and powerful tool for plant breeding programmes in genetic mapping, variety identification and germplasm studies. Isozymes and restriction fragment length polymorphism (RFLPs) markers were until recently the most used markers. After the discovery of random amplified polymorphic DNA markers (RAPDs) by Williams *et al* (1990) and Welsh and McClelland (1990), this polymerase chain reaction (PCR)-based new class of molecular markers has quickly proved to be an interesting alternative for varietal identification (Caetano-Anollès *et al*, 1991; Hu and Quiros, 1991; Welsh *et al*, 1991), phylogenetic studies (Tulsieram *et al*, 1992), and mapping (Carlson *et al*, 1991; Klein-Lankhorst *et al*, 1991; Martin *et al*, 1991; Micheltore *et al*, 1991) because of the simplicity of the technique, the low quantity of target DNA necessary for genetic analysis and the possibility of automatization.

In the *Helianthus* genus and in the sunflower *Helianthus annuus*, isozymes have been used for line characterization (Quillet *et al*, 1991) and phylogenetic studies (Rieseberg *et al*, 1991). RFLPs have been reported in phylogenetic studies (Gentzbittel *et al*, 1992) and line descriptions (Gentzbittel *et al*, 1994a). A RFLP linkage map of cultivated sunflower has recently been constructed in our laboratory (Gentzbittel *et al*, 1994b). Rieseberg *et al* (1993) have also reported the construction of a genetic map by using RAPD markers in *H. anomalus*, a diploid species possibly derived *via* hybridization between *H. annuus* and *H. petiolaris*. However, RAPD strategy has never been explored in the cultivated sunflower.

We report here the application of RAPD markers for the intraspecific identification of cultivated inbred lines of sunflower (*H. annuus* L.) and some molecular characteristics of RAPD fragments.

MATERIALS AND METHODS

Plant material

Seventeen cultivated sunflower (*H. annuus* L.) inbred lines (HA89, H52, 2603, 89B1, GH, SD, 227362, HA124, HA300, RHA274, PB3, PAC2, PX7, RHA266, RHA345, CANP3 and PAT4) obtained from F Vear (INRA, Clermont-Ferrand, France) were used for RAPD analysis.

DNA extraction

DNA was extracted from sunflower green leaves according to the CTAB protocol of Rogers and Bendich (1985) with minor modifications.

RAPD analysis

PCR amplification conditions were defined after testing various Taq polymerase sources (Appligene, Boehringer, Perkin-Elmer, Promega) and concentrations (from 0.5 to 2 U/μl), MgCl₂ concentrations (from 1 to 10 mM), template DNA concentrations (from 50 to 400 ng / 50 μl amplification mix) and amplification cycles. The following protocol was chosen. Amplification reactions were performed in 50 μl aliquots containing 10 mM Tris-HCl, pH 9, 50 mM KCl, 3 mM MgCl₂, 0.2 mg/ml gelatin, 100 μM of each dNTP, 0.2 μM primer (10-mers from Operon Technologies Inc, Alameda, CA, USA), 100 ng DNA and 1 unit of Taq DNA polymerase Appligene. A Perkin-Elmer Cetus DNA thermal cycler 9600 was used, programmed for 1 initial denaturation step for 5 min at 93°C, 40 cycles of amplification of 1 min at 91°C, 1 min at 36°C and 2 min at 70°C, and one terminal extension step of 5 min at 70°C. Amplification products were analyzed after electrophoresis in 1.4% agarose gels and ethidium bromide staining.

Conversion of RAPD amplification product to RFLP probes

Some of the RAPD fragments were excised from low melting agarose gels, purified by a Magic PCR DNA purification system (Promega) then used as probes for RFLP analysis. Procedures for RFLP analysis were as described by Gentzbittel *et al* (1994a). DNAs from the 17 sunflower lines were digested with either *Hind*III or *Eco*RI.

RESULTS AND DISCUSSION

Screening of random primers useful for characterization of sunflower inbred lines

One hundred decamer primers of random sequences (Operon kits C, E, J, M and S) were tested with DNA from 4 lines (RHA266, HA300, HA89 and PAC2). The amplified DNA fragments obtained ranged from 200 to 3 000 bp. Usually, more than 10 bands were detected. However, most were weakly amplified (faint bands), so the number of major bands varied from 1 to 4. Some major bands were common to

the 4 lines while others were specific to 1 line. This demonstrates that polymorphisms between amplification products of the 4 lines can be detected. Forty percent of the primers detected polymorphism (at least 1 line different from the others), 29% were monomorphic, 19% produced no amplification and 12% gave smears.

Low reproducibility is known as the major problem using RAPD markers. The experiments described above were repeated 3 times with the same DNA extract. The primers giving the same pattern for the 3 replicates were selected and will be called RAPD markers in the following

sections. Among the 100 primers tested (Operon kits C, E, J, M and S), only 14 produced RAPD markers with polymorphic and reproducible signals: primers C16, E06, E07, E08, E19, J09, J14, J19, M02, M06, M13, M16, M18 and S18.

Some RAPD markers were tested to characterize 17 inbred lines of cultivated sunflower. As shown in figure 1, the bands obtained with only 4 RAPD markers allow us to distinguish the lines from one another. It is thus possible to select a set of random primers to characterize different lines of cultivated sunflower.

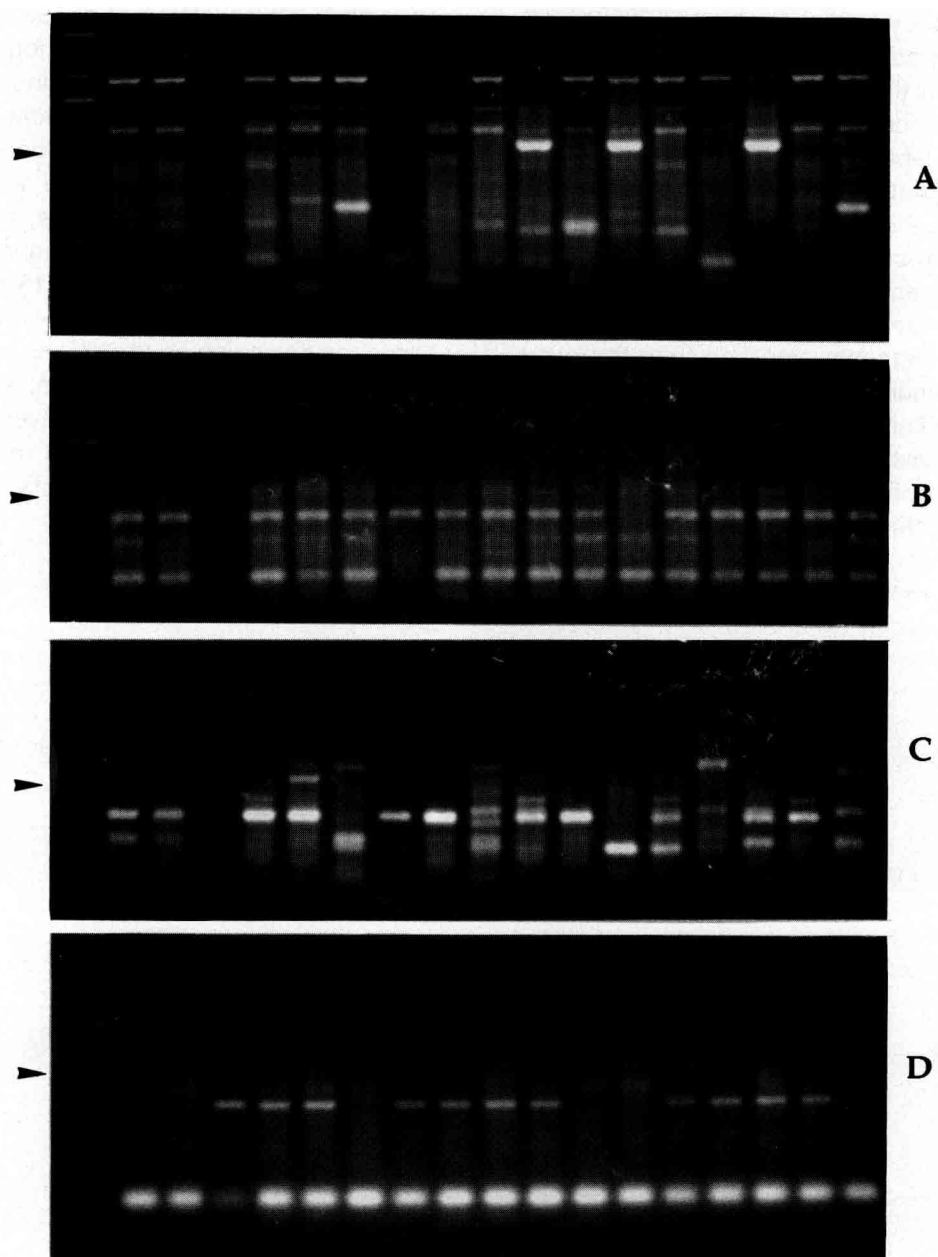


Fig 1. Characterization of inbred lines of sunflower with RAPD markers; amplification of DNA from 17 inbred lines of sunflower is shown. From left to right: molecular size markers (BRL DNA ladder; the arrow indicates the position of the 1 kb fragment), amplification with DNAs from inbred lines PB3, H52, 2603, 89B1, GH, Z2736.2, HA124, HA300, RHA274, HA89, PAC2, PX7, RHA266, RHA345, CANP3 and PAT4. The primers used were: A) OPJ01; B) OPJ04; C) OPE17; and D) OPC16.

Molecular analysis of RAPD fragments

Genetic studies have shown that RAPD fragments are often dominant markers (Rafalski *et al*, 1991). In this case, for each locus the RAPD assays detect the presence of only 1 allele, that giving an amplification; the absence of an amplified band on the other genotypes represents all the other alleles at that locus that fail to prime amplification. To assess whether it is also the case for RAPD markers on sunflower lines, electrophoresis gels of RAPD amplification products have been made in duplicate. One of the gel was blotted onto a Hybond N⁺ filter; some fragments of the other gel were excised, labeled with ³²P and then used as probes onto the filter (data not shown). When the probe was a monomorphic fragment (identical from 1 line to another), this fragment was labeled at the same position for all the lines, indicating that the segment of DNA amplified was the same for the different genotypes. In contrast, when the probe was a polymorphic fragment (present on one line but not observed for the others at the same position) none of the fragments amplified from the other lines were hybridized. These results indicate that the polymorphism revealed by RAPD markers between sunflower lines agrees with the 'present vs absent' type. For 1 locus only 1 allelic form is amplified; the others are not. Genetic studies on

F2 segregants confirm the dominant status of RAPD markers (data not shown).

To study the type of sequences amplified with random primers, we have used random amplified fragments as probes for Southern blot analyses. Amplified fragments were excised from gels after electrophoresis, radiolabeled then hybridized to filters containing DNA from 17 lines digested with 2 restriction enzymes, *Eco*RI or *Hind*III. When polymorphic fragments were used as probes, in most cases they gave smears on Southern blots (data not shown). This indicates that these amplified products contained repeated sequences present in numerous loci in the genome, as observed in lettuce (Paran *et al*, 1993; Kesseli *et al*, 1994). Only one of the 20 polymorphic fragments tested in this study gave a simple but monomorphic signal on Southern blots, as shown in figure 2A. When monomorphic bands were used as probes, they often produced single hybridization signals on Southern blots, indicating that they correspond to unique sequences in the sunflower genome. Three of the 13 monomorphic fragments used revealed polymorphism between lines, as shown on figure 2B with the example of the fragment OPS18-3. Thus, some random amplified fragments can be converted into RFLP probes, with the unexpected result that only monomorphic RAPD fragments can generate polymorphic RFLP probes.

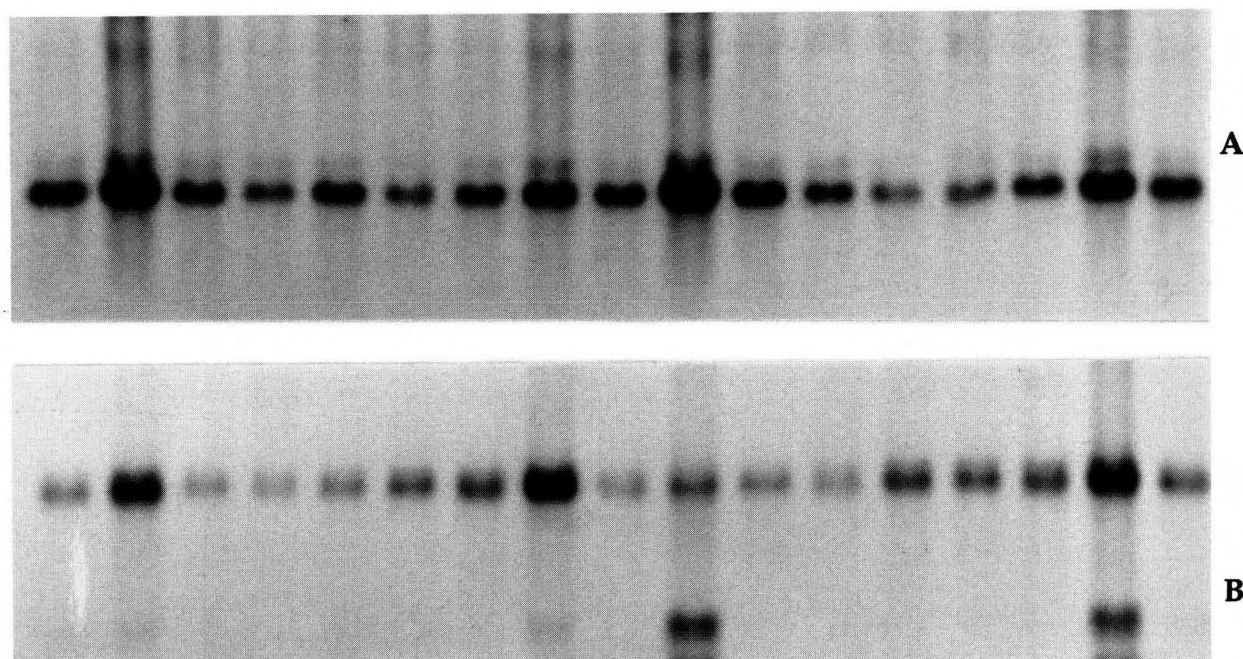


Fig 2. Use of RAPD amplification products as RFLP probes. DNAs from sunflower lines were digested with *Eco*RI, separated on a 0.8% agarose gel and blotted onto Hybond-N⁺. Southern blots were hybridized with radiolabeled PCR products obtained with random primers. From left to right: hybridizations on digests from lines: PB3, H52, 2603, 89B1, GH, SD, Z2736.2, HA124, HA300, RHA274, HA89, PAC2, PX7, RHA266, RHA345, CANP3 and PAT4. **A)** Hybridization with the polymorphic PCR product OPJ04-2 as probe; and **B)** hybridization with the monomorphic PCR product OPS18-3 as probe.

CONCLUSION

In previous work, we showed that RFLP markers can be used to characterize species of the *Helianthus* genus (Gentzbittel *et al*, 1992) or inbred lines of the cultivated sunflower *H annuus* (Gentzbittel *et al*, 1994a). In the present work we showed that RAPD markers, described by Rieseberg *et al* (1993) as usable for genetical mapping in interspecific crosses, can be used for discrimination between inbred lines of sunflower.

Compared with RFLP markers, RAPD markers present 2 deficiencies. The first is the weak reproducibility from 1 experiment to another, as discussed by Ellsworth *et al* (1993). Indeed, we observed that only a third of the random primers producing a simple and polymorphic signal gave reproducible amplifications. Thus, only 14% of the random primers tested can be used as reproducible polymorphic markers. However, as hundreds of random primers are potentially available, it is possible to find numerous useful RAPD markers. In addition, our results show that a small number of markers is sufficient to some applications such as discrimination between inbred lines. To obtain more precise and reproducible markers corresponding to amplified RAPD products, it is also possible, after partial sequencing of the ends of the amplified fragments, to get specific primers for each end. Such PCR-amplified fragments were called SCARs (sequence-characterized amplified regions) by Paran and Michelmore (1993).

The second deficiency of RAPD markers, when used for genetic studies such as construction of chromosome maps, is that they reveal polymorphism of the 'presence/absence' type and segregate as dominant alleles, as reported in broccoli and cauliflower (Hu and Quiros, 1991), lettuce (Michelmore *et al*, 1991) or canola (Deragon and Landry 1992). In this study we also observed the 'presence/absence' type of polymorphism with RAPD fragments obtained from sunflower inbred lines. Thus in crosses it is impossible to distinguish between heterozygous and homozygous genotypes with the amplified fragment. RAPD markers are therefore less informative than codominant RFLP markers (Roy *et al*, 1992).

In spite of these disadvantages, RAPD markers may be useful for genetic studies of inbred lines of sunflower. Experiments are simple and fast to perform, they do not need radioactivity and only small amounts of plant material are required.

Our results show that RAPD markers can be used for characterization of lines, allowing classification and phylogenetic studies. Furthermore, they can provide additional markers to the sunflower RFLP chromosome map in progress in our laboratory (Gentzbittel *et al*, 1994b). Indeed, we have demonstrated here that RAPD markers reveal polymorphism on DNA segments including highly repeated sequences, whereas RFLP markers correspond to unique or weakly repeated sequences (Gentzbittel *et al*, 1994a). RAPD markers may thus map on chromosomal segments distinct from those where RFLP markers are localized.

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