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Comparing AFLP, RAPD and RFLP markers for measuring genetic diversity in melon

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Abstract Three different types of molecular markers, RAPD, AFLP and RFLP were used to measure genetic diversity among six genotypes of *Cucumis melo* L. Each line represented a different melon genotype: Piel de Sapo, Ogen, PI161375, PI414723, Agrestis and C105. A number of polymorphic RAPD, AFLP and RFLP bands were scored on all materials and the genetic similarity measured. Clustering analysis performed with the three types of markers separated the genotypes into two main groups: (1) the sweet type, cultivated melons and (2) the exotic type, not cultivated melons. While the data obtained suggest that all three types of markers are equally informative, AFLPs showed the highest efficiency in detecting polymorphism.

Key words Melon · AFLP · RFLP · RAPD · Genetic similarity

Introduction

Melon (*Cucumis melo* L.) is a vegetable crop of great importance in tropical and subtropical climates. So far studies with molecular markers in melon have involved the search for polymorphism among melon germplasm to clarify genetic relationships and the organisation of taxa (Katzir et al. 1995, 1996; Kovalski et al. 1995). Molecular markers have also been used for the construction of molecular maps (Baudracco-Arnas and Pitrat, 1996; Wang et al. 1997).

Different types of genetic markers have been used to assess genetic diversity in melon: initially, isozymes (Perl-Treves et al. 1985; Staub et al. 1987) and later restriction fragment length polymorphisms (RFLPs) (Neuhausen 1992), random amplified polymorphic DNA (RAPDs; Garcia et al. 1998) and simple sequence repeats (SSRs; Katzir et al. 1996). The effectiveness of isozymes is limited by the low number of detection systems available and low isozymic variation in *C. melo* germplasm. On the contrary, RFLP studies (Neuhausen 1992) proved that this type of marker was sufficient to classify 44 *C. melo* lines. More recently, Perl-Treves et al. (1998) reported an intraspecific classification of melon using RAPDs and ISSRs in 54 melon accessions in which melon was clearly separated into two subspecies: sweet types (*inodorus, cantalupensis* and *flexuosus*) and exotic types (*momordica, agrestis, conomon, chito* and *dudaim*). Garcia et al. (1998) have also successfully used RAPDs for assessing diversity in melon; however to date there is no report of the use of amplified fragment length polymorphism (AFLP) technology to determine genetic relationships among melon germplasm.

Comparisons of the performance of several types of molecular markers in measuring genetic diversity have been carried out in several plant species. Pejic et al. (1998) studied 33 maize inbred lines using RFLP, RAPD, SSR and AFLP markers and concluded that both the SSR and the AFLP technologies can replace RFLPs. Lu et al. (1996) studied ten pea genotypes with RFLP and polymerase chain reaction (PCR)-based markers and obtained the same genetic trees with all markers. Russell et al. (1997) used RFLPs, AFLPs, RAPDs and SSRs to compare genetic variation among 18 cultivated barley accessions. Other studies have also been reported in maize (Beaumont et al. 1996) and soybean (Lin et al. 1996).

The goal of the study reported here was to compare three different types of molecular markers to establish which of them is more suitable to measure genetic diversity in our melon germplasm. We were interested in obtaining segregating populations for important agronomic traits but at the same time choosing the right parents with the highest level of polymorphism at a molecular level. Six melon accessions of different origins were used for that purpose. The six accessions belong to dis-

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tinctly different genetic backgrounds: they cover the melon groups *agrestis, inodorus, conomon,* and *momordica* (Perl-Treves et al. 1998). The power of each marker type to detect polymorphism and the similarity measures obtained within each system are presented.

Materials and methods

Plant material

The melon accessions used in this study were the following: the Spanish cultivar Piel de Sapo line T-111, PI161375 from South Korea, PI414723 from India, C105 from Zimbabwe, Agrestis from India and the Ogen line LP125 from Israel. The different lines were provided by either Semillas Fitó SA or the Estación Experimental 'La Mayora' (CSIC) in Malaga, both in Spain. Seeds were germinated and maintained *in vitro* for leaf DNA extraction.

DNA extraction

Total genomic DNA was isolated from young leaves as described by Doyle and Doyle (1990) with a few modifications intended to improve the quality of the DNA: two consecutive extractions with chloroform-isoamyl alcohol (24:1) were performed followed by an additional wash with 5 *M* NaCl for 20 min at –20°C, before final precipitation, to reduce the presence of polysaccharides. DNA used for RFLPs was further purified through a CsCl gradient (Sambrook et al. 1989).

RAPD markers

The protocol for RAPD analysis was adapted from that of Williams et al. (1990). The volume of the final reaction (25 µl) was composed of 1 × buffer (50 m*M* KCl, 10 m*M* TRIS-HCl pH 8.3, 1.5 m*M* $MgCl_2$, 0.001% gelatine), 0.44 m*M* $MgCl_2$, 0.1 m*M* dNTPs, 30 ng of primer, 1.5 U Ampli*Taq* (Perkin Elmer-Applied Biosystems) and 10 ng of template DNA. Primers belonged to the Operon series A to Y. Amplifications were performed in a Perkin Elmer 9600 thermocycler with an initial denaturing step of 1 min at 94°C followed by 45 cycles of 10 s at 94°C, 10 s at 35°C and 1 min at 72°C and a final extension step of 2 min at 72°C. PCR products were run at 120 V on a 2.5% agarose gel, and DNA bands were visualised by ethidium bromide staining.

RFLP markers

Probes used in this study came from two different DNA libraries: a genomic *Hin*dIII library constructed on the pBluescript vector (Stratagene) from Piel de Sapo line T-111 and a cDNA lambda-ZAP library (Stratagene) made from young leaves of PI161375. Five micrograms of genomic DNA from the six different lines was digested with the restriction enzyme *Eco*RV, run on 0.9% agarose gels and blotted onto Hybond N+ membranes. Probes were radiolabelled according to the random priming method (Sambrook et al, 1989). Membranes were prehybridised in 0.5 *M* Na_2HPO_4, 0.5 *M* NaH_2PO_4, 7 *M* SDS, 10 mg/ml BSA and 0.05 mg/ml denatured salmon sperm DNA at 65°C for 2–3 h and subsequently hybridised in the same buffer with the labelled probe at 65°C overnight. Washes were performed at 65°C with 2 × SSC, 0.1% SDS and 1 × SSC, 0.1% SDS for 20 min each time, and membranes were exposed to AGFA Curix RP2 film for 2–3 days.

AFLP

DNA was double-digested with *Eco*RI and *Mse*I following the protocol of Vos et al. (1995). Resultant fragments were ligated to adapters specific for the *Eco*RI and *Mse*I restriction sites. A preselective amplification was carried out with *Eco*RI+A and *Mse*I+C primers, and the PCR product was then diluted 15-fold with water and used as template for the selective amplifications using both *Eco*RI+3 and *Mse*I+3 primers. *Eco*RI+3 primers were fluorescently labelled with yellow, green and blue dyes (Perkin Elmer-Applied Biosystems). Labelled fragments were run on an Abi-Prism 310 Automated DNA Sequencer (Perkin Elmer-Applied Biosystems) and analysed using GENESCAN Analysis Software 2.0. Primer combinations used in this work were the following: MseCTC-EcoAAC, MseCTC-EcoAAG, MseCTC-EcoACA, MseCTA-EcoACC, MseCTA-EcoACG, MseCTA-EcoACT, MseCAT-EcoAAC, MseCAT-EcoAAG, MseCAT-EcoACA, MseCAC-EcoAAC, MseCAC-EcoAAG and MseCAC-EcoACA.

Data analysis

DNA fragments were scored as present (1) or absent (0) for each of the markers. Genetic similarity was measured with the SIMQUAL programme, which computes similarity coefficients for qualitative data using the Dice similarity index. The Dice similarity index calculates the similarity between two samples *i,j* with the formula $GS(i,j)=2a/(2a+b+c)$ where $GS(i,j)$ is the similarity coefficient between samples *i* and *j, a* is the number of bands shared between *i* and *j, b* is the number of bands present in *i* and absent in *j* and *c* is the number of bands present in *j* and absent in *i*. Similarity trees were produced by clustering the similarity data with the unweighted pair group method using arithmetic averages (UPGMA) and the SAHN clustering programme. Comparison among the data produced with the different types of markers was done using the Mantel test (Mantel 1967), a randomisation procedure that compares the correlation between two matrices with the correlation between one of these and randomisations of the other. All analyses were performed with the NTSYS package 7.0 (Rohlf 1997).

Results

RAPD analysis

A total of 500 primers were tested with the six melon accessions. This resulted in 204 primers (40.8%) showing at least 1 consistent polymorphic band among the six genotypes. The total number of polymorphic bands scored was 364, which represents an average of 0.73 polymorphic bands per total number of primers used (Table 1).

Table 1 Characteristics of the RFLP, AFLP and RAPD markers used in analysing the genetic variability of six melon genotypes

	Total number of probes/primers	Number of polymorphic probes/primers	Percentage of polymorphic probes/primers	Number of polymorphic bands	Number of polymorphic bands per total number of probes/primers
RFLP	82	47	57.3	121	1.47
RAPD	500	204	40.8	364	0.73
AFLP	12	12	100	181	15.08

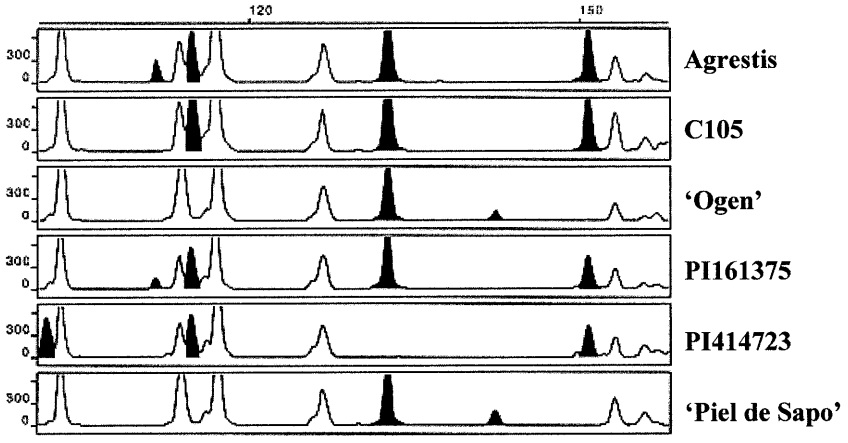

Fig. 1 AFLP pattern obtained with the GENESCAN software on the six melon accessions using the primer combination *Mse*I-CAT /*Eco*RI-AAC. Polymorphic peaks among the six lines are shown in *black*. The peak intensity value is on the *left*, and the size of the peaks in base pairs is at the *top*

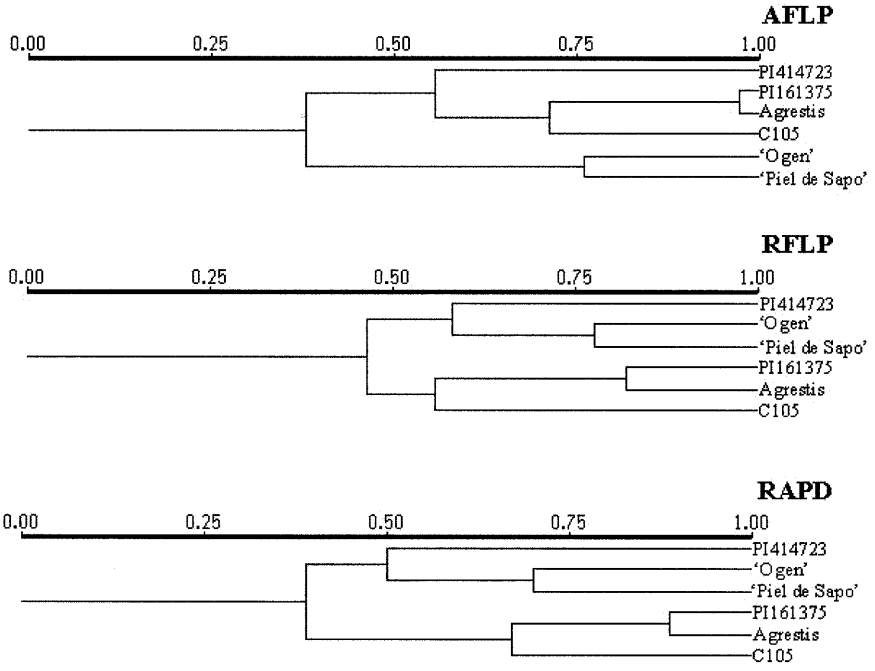

Fig. 2 Dendrograms obtained on the six melon accessions (PI414723, PI161375, Agrestis, C105, Ogen and Piel de Sapo) with AFLPs, RFLPs and RAPDs. Dendrograms were obtained using the TREE option on the NTSYS-PC software. The scale is the genetic similarity index (GS) with a maximum value of 1.0

AFLP analysis

The six melon accessions were analysed with 12 AFLP primer combinations, all of which showed at least 1 polymorphic fragment. The total number of bands screened was 783, of which 181 (23.1%) found a polymorphic pattern among the melon lines. The number of polymorphic bands per total number of primer combinations used was, on average, 15.08 (Table 1). A representation of the AFLP pattern obtained for all genotypes with 1 of the primer combinations, *Mse*I-CAT/*Eco*RI-AAC, is shown in Fig. 1.

RFLP analysis

A total of 82 probes were used in this study, 69 cDNA and 13 genomic clones. DNA was digested with the restriction enzyme *Eco*RV. This enzyme showed a higher level of polymorphism than other restriction enzymes previously used, such as *Eco*RI, *Hind*III, *Mva*I and *Bam*HI (data not shown). Forty-seven probes (57.3%) evidenced at least 1 polymorphic band among the six lines. The total number of scored bands was 195, of which 121 were polymorphic (62%). The average number of polymorphic bands per total number of probes used was 1.47 (Table 1). The cDNA probe MC69 gave 11 RFLP fragments, of which 9 were polymorphic among all materials.

Genetic similarity

The same number of informative RAPD, AFLP and RFLP markers was compared: 107 polymorphic bands. These bands were randomly chosen from the total number of polymorphic bands obtained with each type of marker (Table 1). The genetic similarity index was calculated using the Dice coefficient among the six melon genotypes, and the UPGMA method allowed the clustering of the lines (Fig. 2). Very similar clustering pictures

were obtained with the results of RAPD, AFLP and RFLP markers, with the only exception being accession PI414723, which appears to be included in a different group when the analysis was performed with the AFLP bands. There are two main groups: the first contains the Piel de Sapo and Ogen genotypes; the second group contains accessions PI161375, Agrestis and C105. PI414723 is clustered with the PI161375 group when using AFLPs, with a similarity value of 0.52; with RFLP and RAPD markers PI414723 is grouped with Ogen, with a similarity value of 0.56 and 0.50, respectively.

Correlation within the three type of marker methods

A comparison of the data obtained with the three types of markers was made. The values of the Mantel test correlation showed a good fit of the data using any of the marker types. The *r* correlation value was 0.79 between AFLPs and RFLPs, 0.91 between AFLPs and RAPDs and 0.90 between RAPDs and RFLPs. These data indicate that the genetic similarity index obtained with each of the marker types in each of the genotypes shared a good correlation, and thus the results are comparable.

Discussion

Six melon genotypes from very different genetic origins were fingerprinted with RAPD, AFLP and RFLP markers. The genetic similarity values obtained with all types of markers were equivalent and the dendrograms obtained were analogous with the results of RAPDs and those from RFLPs. AFLPs gave a slightly different cluster with accession PI414723, which was placed in a different group.

In the last 10 years RFLPs have been the marker of choice for cultivar identification and fingerprinting. We have observed that the percentage of polymorphic probes (57%) is high among the group of melon genotypes using the restriction enzyme *Eco*RV. Neuhausen (1992), with different germplasm, found that 33% of the RFLP probes used among seven *C. melo* accessions were polymorphic. However, the development of RFLP technology is tedious due to the large amounts of DNA and the special technical equipment needed. Moreover, automation of the technique is difficult.

More recently, RAPDs have been introduced for measuring genetic relationships in many plant species. The easiness of the method, which only requires PCR technology, has determined its replacement of RFLPs for genetic variability assessment. However, the low reproducibility of RAPDs (Karp et al. 1997) introduces a problem in their use for cultivar identification and other marker applications. It is worth noting that putatively similar bands originating from RAPDs in different individuals are not necessarily homologous although they may share the same size in base pairs. This situation may lead to wrong results when calculating genetic relationships. We observed polymorphic bands in 40.8% of the primers used and estimated that 18% of the total number of observed bands were polymorphic. Katzir et al. (1996) found that 38% of the RAPD primers used were polymorphic within a sample of eight melon varieties. Baudracco-Arnas and Pitrat (1996) estimated that 18.3% of the total number of bands observed between the melon lines, Védrantais and PI161375, were polymorphic. These data are consistent with the results we obtained. Garcia et al. (1998) described 49% polymorphic bands in a survey of six representative lines of sweet melon germplasm. This increased percentage can not be compared with our results because the primers used in their study were previously selected non-randomly from a higher number of primers.

We introduced AFLP markers for measuring genetic distances in melon germplasm in order to see if this technique was able to substitute for RAPDs and RFLPs. Although the AFLP technology is sophisticated, requires special equipment and is more expensive, it is reproducible and low amounts of DNA are needed when compared with both RAPDs and RFLPs, respectively. The level of polymorphism (polymorphic bands/total bands) detected with AFLPs was substantially lower than that seen with RFLPs (23.1% vs. 62%) but similar to RAPDs (23.1% vs. 18%). Russell et al. (1997) also reported a lower percentage of polymorphic bands with AFLPs and RAPDs than with RFLPs (46.8% and 66.3% vs. 83.2%) in a survey of 18 cultivated barley accessions. However the efficiency of AFLPs versus RAPDs and RFLPs was much higher because 15.08 polymorphic bands were obtained per primer combination. This value was as low as 1.47 for RFLPs and 0.73 for RAPDs.

The UPGMA analysis gave similar results with the three types of markers. The clusters were similar and two main groups were found as expected: one of them contained the sweet melon types Piel de Sapo and Ogen, and the second one the wild melon accessions PI161375, Agrestis and C105. The accession PI414723 was placed in the second group with AFLPs and in the first group with RAPDs and RFLPs, but in both cases with similarity indexes lower than 0.56. We can not rule out the possibility that if the number of accessions in the analysis were increased the clustering of PI414723 would be more precise, because other lines more similar to PI414723 should form a new cluster. Accession PI414723 is known to belong to var. momordica. Recently, Perl-Treves et al. (1998) clustered PI414723 in a group of exotic varieties that also contains members of var. agrestis and var. conomon using RAPD and ISSR markers. This is consistent with the results we obtained with AFLP markers where PI414723 is clustered in the Agrestis and PI161375 group.

The AFLP analysis also included the melon cultivar Charentais, which belongs to var. cantalupensis. In the cluster obtained with Charentais and the previous six genotypes, the former was clustered in the group of sweet melons with Piel de Sapo and Ogen (not shown), thus confirming the results obtained by Perl-Treves et al.

(1998). Melon germplasm would be divided in two main groups, one of them containing the sweet type melons (Piel de Sapo, Ogen, Charentais) and the second group with the exotic melons (PI161375, PI414723, C105 and Agrestis).

These results confirm the usefulness of AFLP technology for cultivar fingerprinting in cucurbit species. The efficiency of the AFLP method is much higher than that of other marker types, with the reproducibility of the AFLP markers making them especially more attractive than RAPDs. However, our results clearly show that any marker method is optimal for genetic similarity studies in melon germplasm, although more data are needed, especially if the germplasm to be studied contains lines belonging to the same variety type. Garcia et al. (1998) reported a RAPD analysis of members of the Piel de Sapo and Galia types and showed that the RAPD technique was able to distinguish members of those inbred groups. On the other hand, preliminary data in our group suggest that only 6 AFLP primer combinations are needed to fingerprint 20 melon breeding lines belonging to the Piel de Sapo group (unpublished).

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