ASSESSMENT OF GENETIC VARIATION WITHIN DATE PALM (*PHŒNIX DACTYLIFERA* L.) USING AMPLIFIED FRAGMENT LENGTH POLYMORPHISM (AFLP) - GENOTYPING OF APOMICTIC SEEDLINGS AS A CASE STUDY

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INTRODUCTION

Date palm (*Phonics dactylifera* L.) plays an important role in the socioeconomic stability of oases regions in north Africa. The Tunisian palm groves count about 4.282.000 palm trees, of which 56 % is Deglet Nour variety (GID, 1999). However, this cultivar is very sensitive to the Bayoud disease, imposing a serious threat to the Tunisian palm groves. As a consequence, new approaches for the mass propagation of date palm, more efficient than offshoot propagation, have to be developed. Apomixis, defined as the production of seeds without fertilization, has been described in angiosperms and allows the clonal multiplication of hybrid genotypes, but spontaneous apomixis has not been described in date palm.

However, the treatment of non-pollinated date palm female inflorescences by gibberellic acid (GA3) produces diploid plants whose origin is assumed to be apomictic (Ben Abdallah, 2000; Ben Abdallah *et al.* 2000), although the true-to-typeness has to be assessed.

The present paper describes the utilization of the AFLP (Amplified Fragment Length Polymorphism) technology in order to detect genetic polymorphism in date palm and to analyze the genetic relationship between the parental cultivar and seed progenies obtained by GA-induced apomixis.

MATERIALS AND METHODS

Plant material

This work used Deglet Nour as the female cultivar (Deglet nour), a pollinator genotype (T23, from INRAT collection), F1 hybrid plants and plants obtained from the seeds derived from the GA3 treatment of non-

pollinated female inflorescences. Both of F1 hybrid and apomictic plants are 2 years old. The GA treatment used concentrations of 30 mg/l, 60 mg/l, the corresponding seedlings (AG30; AG60) being separately analyzed.

Extraction of total DNA

Total DNA was extracted from 1g of leaves (kept at –70°C after being crushed to powder in liquid nitrogen) according to Aitchitt *et al*. (1993). As a minor modification, the DNA pellet was washed with 70% ethanol (v/v) after precipitation in the presence of sodium acetate and absolute ethanol. DNA quality was examined by electrophoresis in 0.8% agarose, and DNA concentration was quantified comparing the fluorescence intensities of the ethidium bromide stained samples to those of molecular weight marker standards (200 and 1000 bp DNA ladder).

AFLP assay

Digestion-ligation

Total DNA (60 ng) was incubated for two hours at 37°C with 1.25U of each restriction enzyme EcoRI and MseI, in a final volume of 12.5 µl containing 2.5 µl of 5X restriction digestion buffer. At the completion of the digestion reaction, the restriction enzymes are inactivated by incubation at 70°C for 15 min.

Immediately following the restriction enzyme inactivation step, 12 µl of an adaptor-ligation solution and 1U of T4 DNA ligase is added directly to micro centrifuge tubes containing the digested DNA and incubated for 2 hours at 20°C.

Preselective amplification and target sequences

Briefly, 2.5µl of a 1:10 diluted portion of the adaptor/ligation reaction mixture is mixed with 20µl of preamp primer solution; 2.5µl of 10X PCR buffer for AFLP and 0.5U of Taq DNA polymerase in storage buffer (1U/µl).

The reaction is a 20 cycle event performed in a Techne model PHC-3 thermocycler using the following parameters: 30s denaturation at 94°C, 1min annealing at 56°C, 1min elongation at 72°C.

A combination of two primers, one for the EcoRI adapter (5'-CTCGTAGACTGCGTACC-3'-**A**) with one selective nucleotide (indicated in bold after the hyphen) and another for the MseI adapter (**C**-3'-TACTCAGGACTCAT-5') with one selective nucleotide (indicated in bold) were used for the preselective amplification of EcoRI-MseI fragments.

Primer Labeling and selective amplification

Primer labeling was performed by phosphorylating the 5' end of the EcoRI primers with [γ^{-33}P]ATP and T4 polynucleotide kinase by mixing 5 µCi of γ^{-33}P-ATP (1µCi/µl with 2 Ci/mmol), 10U of T4 polynucleotide kinase and 5µl of T4 5X kinase buffer (350 mM Tris-HCl (pH 7,6), 50 mM $MgCl_2$, 500 mM KCl, 5 mM 2-β-mercaptoethanol).

^{33}P labeled primers are preferred because they give better resolution of the PCR products on the gels. Also, the reaction products are less prone to degradation due to autoradiolysis.

An aliquote of 5 µl of the 1/50 diluted pre-amplification (in TE1X) was amplified in a final volume of 20 µl containing 30 ng MseI primer; 14 ng labeled EcoRI primer (0,5µl of the primer labeling reaction) having 2 selective nucleotides at the 3' ends; dNTPs associated to MseI primer; 0,5µl of 5.0U/µl Taq DNA polymerase and 2µl of 10X PCR buffer (0,1M Tris-HCl (pH 8,3), 0.5 M KCl, 1.5 mM $MgCl_2$).

The selective amplification reaction performed using a Techne model PHC-3 thermocycler. Starts with one cycle at 94°C for 30 s, 65°C for 30 s; and 72°C for 60 s. Once this cycle done, the annealing temperature was decreased each cycle 0.7°C during 12 cycles. This gives a touch down phase of 13 cycles. The 13th cycle is bound to a set of 23 cycles using the following parameters: 30s denaturation at 94°C, 30s annealing at 56°C and 1min elongation at 72°C.

Denaturing polyacrylamide gel analysis

At the conclusion of the selective amplification reaction, the AFLP products are separated electrophoretically.

The products are prepared for electrophoresis by mixing 20 µl of each sample with an equal volume (20µl) of formamide dye (98% formamide, 10mM EDTA, 0,015% xylene cyanol, 0.015% bromophenol blue), denaturing at 95°C for 3min, followed immediately by chilling on ice. AFLP products are electrophoresed in a 6% denaturing polyacrylamide gel. It was prepared with 46.7% (w/v) urea, 15% acrylamide solution and 1XTBE buffer.

Gels were pre-electrophoresed 30min at constant power:80W (45mA). Five microlitres of each sample were loaded into wells. Electrophoresis was performed at constant power (110W), for~ 2 hours.

RESULTS

Primer-pairs were first selected, which detected polymorphisms within genotypes used. Out of 9 primer-pairs tested, 4 were selected because they were reproducible giving 154 polymorphic bands (30% of the total) (Table 1). The comparison of the AFLP profiles was done using 14 genotypes (pollinator genotype, female genotype, F1 with 2 genotypes, AG30 and AG60 plants each with 5 genotypes).

Table 1. Selective AFLP primer-pairs and respective number of generated bands

Selective AFLP primer-pairs (EcoRI- / MseI-)	Number of amplification products**	Number of polymorphic bands
AGG / CAA*	81	**62**
AGG / CTA*	76	**53**
AGG / CTT	55	33
AGG / CAG*	33	**13**
AGG / CTG	52	18
ACG / CAC	20	9
ACC / CTC*	41	**21**
ACC / CTA	67	20
AAC / CAA	88	19
Total	513 (100%)	248 (48%)

** selected primer-pair*

*** As scored by visual comparison of bands between 80 and 330 bp on ^{33}-P labeled gels.*

The 9 primer-pairs generated distinctive products with an average of 57 bands in the range of 80-330 bp. Bands outside this range were not considered. Only bands that were consistently reproduced were considered for the analysis. Bands with the same mobility were considered as identical fragments, receiving equal values, regardless of their radioactive intensity.

When multiple bands in a region were difficult to resolve, data for that region of the gel was not included in the analysis. This was done to avoid scoring fragments as identical when they were actually different.

Genetic distance analysis indicated that the use of four primer-pairs generated reliable dendrograms (i.e. additional primer-pairs did not change the grouping of the genotypes).

Interestingly, the apomictic offsprings of AG30 proved to be off-type where compared to the mother plant (Deglet Nour, figure 1).

Figure 1. AFLP fingerprinting of apomictic seedlings and their mother cultivar Deglet nour. Only part of the gel, with fragments between 80 and 330 base-pairs is shown. The bands were detected on 6% denaturing polyacrylamide gel using ^{33}P-labelling and the primer-pair EcoRI-AGG/ MseI-CAA. Based on the obtained patterns, the proportion of polymorphic fragments (as shown by the arrows) is determined and used for calculating Nei genetic distances among the genotypes.

The hybrids F1, with 2 genotypes, AG30 plants, with 5 genotypes and AG60 plants with 5 genotypes form monophyletic groups. The plants obtained with 30 mg/l and 60 mg/l formed separate clusters, suggesting that the dose of the applied GA has an impact on the genetic makeup of the apomictic progeny.

These findings corroborated previous results obtained with other types of markers (RAPD, isoenzymes, Ben Abdallah, unpublished observations) indicating the non-conformity of the apomictic lineage in date palm, as obtained by gibberellic acid treatment.

CONCLUSION

The AFLP technique proved to be an efficient, practical and reproducible tool for the fingerprinting of close genotypes. We suggest that this technique will prove very informative for probing the genetic diversity in date palm. Unexpectedly, seedlings derived from GA-induced apomixis proved to be off-type when compared to the mother plant. Our future work will need to address the apomictic pathway explaining this behavior.

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