Journal of Cell and Molecular Research (2009) 1 (2), 68-71

Application of RAPD (Random Amplified Polymorphic DNA) marker for sex determination of *Pistacia vera* L.

Ali Akbar Ehsanpour* and Lila Arab

Department of Biology, Faculty of Science, University of Isfahan, Isfahan, Iran

Received 22 August 2009 Accepted 19 September 2009

Abstract

Sex determination in Pistacia as a dioecious plant is economically desirable. Identification of the male and female plants has a great value for Pistacia yield production. In the present study, leaf samples from four female plants from cultivars of Pistacia including Akbary, Akbar Aghaei, Fandoghi, and Kalleh Ghochi as well as male plants were used for RAPD PCR amplification. Among 32 primers with 10 mer two primers (FPKI106 and FPK105) were able to discriminate between female and male plants. Few bands were detected in the DNA pattern of male while they were absent in the female individuals. The PCR conditions were reproducible and can be recommended for sex determination of *Pistacia vera* cultivars

Keywords: *Pistacia vera*, RAPD-PCR, sex determination

Introduction

Within the last decade, technological advancements have increasingly supported the use of genetics in determining population diversity of plants. Many molecular techniques are now available, which allow ecologists and other biologists to determine the genetic structure of a wide variety of closely related individuals. DNA markers that are known to be genetically linked to a trait of interest can be used for gene cloning, medical diagnostics, sex determination, and for trait introgression in plant and animal breeding programs (Lynch and Milligan, 1994).

Several different methods including isozyme analyses, restriction fragment length polymorphisms (RFLP), and random amplified polymorphic DNA (RAPD) are used for documenting genetic information. Although isozyme analysis and RFLP are a source of readily obtainable genetic information which is easily reproduced, they often do not show polymorphisms which are necessary to determine variation within a group of genetically similar individuals (Mulcahy, et al., 1993, Williams et al., 1990)

Because RAPD is a relatively straight-forward technique to apply, and the number of loci that can be examined is unlimited, RAPD analysis is viewed as having a number of advantages over RFLP's and other techniques (Lynch and Milligan, 1994), for example, no preliminary work such as probe isolation, filter preparation, or nucleotide sequencing is necessary (Williams et al.,1990). In many instances, only a small number of primers are necessary to identify polymorphism within species (Williams et al., 1990). Indeed, as Mulcahy et al., (1995) report, a single primer may often be sufficient to distinguish all of the sampled varieties. Williams et al. (1990).

The genus Pistacia, in the family Anacardiaceae, contains at least 12 tree and shrub species. P. vera L. (2n=32) the only edible nuts, is cultivated widely in the Mediterranean regions of Europe, Middle East and California. Pistacia breeding programs have been initiated to develop new cultivars (Irish and Nelson, 1989). Dioecy represents an inconvenience to pistachio breeding since the reproductive maturity of pistachio seedlings takes between 5 and 8 years. Currently there is no method for distinguishing between male and female pistachio seedlings prior to flowering. A method to determine the gender of plants before flowering would facilitate breeding and selection by enabling screening for gender at the seedling stage, thereby simplifying the breeding of male and female plants for different objectives, with a saving in time and economic advantages (Bawa, 1980). The presence of sex chromosomes has been claimed for other dioecious angiosperms, but in only a few cases has that claim been documented (Westergaard, 1958; Lewis and John, 1968; Parker and Clark, 1991). Examples include the genera Humulus, Rumex and, perhaps, Cannabis, although the presence of heterochromosomes in the is controversial

* Corresponding author, e-mail: ehsanpou@yahoo.com

(Westergaard, 1958; Durand and Durand, 1990).

Well documented examples include species of the genera Asparagus, Vitis, Spinacia and Mercurialis. In most of the species examined in details, the study of sex determination is complicated by the presence of additional alleles or factors that can modify the effects of the major sex determining genes (Durand and Durand, 1990). Pistacia vera shows perfect dioecy; mature female flowers have no trace of stamens and mature male flowers lack any evidence of female structures (Wannan and Quinn, 1991). This clear differentiation of sexual phenotype, combined with its perennial nature, an increasing economic importance of the crop and recent interest in breeding improved cultivars, makes the P. vera attractive for the study of different aspects of sex determination.

Materials and Methods

Plant material and DNA extraction

At least three fresh leaf segments from 7 years old individual female Pistacia plants cultivars Kalleh Ghochi (K), Akbari (A), Akbar Aghaei (AA), Fandoghii (F), and male plants were harvested from Pistacia field in Ardestan in Isfahan and kept in the freezer.

DNA was extracted according to the CTAB (hexadecyltrimethylammonium bromide) method of Doyle and Doyle (1987) with some modifications. Young leaf tissue (100 mg) was ground to fine powder in liquid nitrogen in 1.5 ml centrifuge tubes and mixed with 0.5 ml of CTAB extraction buffer (100 mM TRIS-HC1, 1.4 M NaC1, 20 mM EDTA, 2% CTAB, 1% PVP, 0.2% mercaptoethanol, 0.1% NaHSO3). The sample was incubated at 65°C for 1 h, mixed with an equal volume of chloroform-isoamyl alcohol (24:1) and centrifuged at 13000 rpm for 5 min in a desktop centrifuge. The aqueous phase was recovered and mixed with equal volume of isopropanol to precipitate the DNA. The nucleic acid pellet washed with 1 ml 10 mM ammonium acetate in 76% ethanol, dried overnight and re-suspended in 100 µl modified TE buffer (10 mM Tris-HCI, 0. 1 mM EDTA). DNA was extracted separately from each individual plant. In all cases extracted DNA (25 ng per 20 µ l reaction mix) was subjected to polymerase chain reaction (PCR) amplification. (All chemicals were supplied from Cinagene Company, Iran)

PCR Amplification: RAPD analysis was performed with 32 decamer primers and amplification reactions were carried out in an Eppendorf Master cycler gradient thermocycler (Eppendorf Netheler-Hinz, Hamburg, Germany). Amplification conditions used were 1 cycle at 94°C for 5 min followed by 39 cycles at 94°C for 30 sec, 30°C for 30 sec and 72°C for 1 min. RAPD reactions (Williams et al., 1990), were carried out in a volume of 20 µ l with 25 ng of genomic DNA, 2 µ M primer, 1 U of *Taq* polymerase, 0.2 mM dNTPs, and 2 mM $MgCl_2$ all from Cinagene Company, Iran. After electrophoresis of PCR products in 0.8% agarose (Cinagene) at 80 V, the gels were stained with 0.5 µ g/ ml ethidium bromide solution and visualized by illumination under UV light.

Results

After screening 32 arbitrary 10-mer Operon primers, we identified FPKI106 and FPK105 primers as being able to differentiate sex type, when tested on four Pistacia male and female plants. These primers generated few bands in male samples which were absent in female plants (Figurs 1 and 2). These markers were reproducible under a broad range of amplification conditions (for example different $MgCl_2$ concentration and different annealing temperature), without any variation in the results. Additional experiments were applied for 4 weeks old seedlings of pistacia cultivars for sex determination. We found the same patterns of DNA bands after PCR amplification using FPKI016 and FPK105 primers for female and male seedlings similar to 7 years old plants.

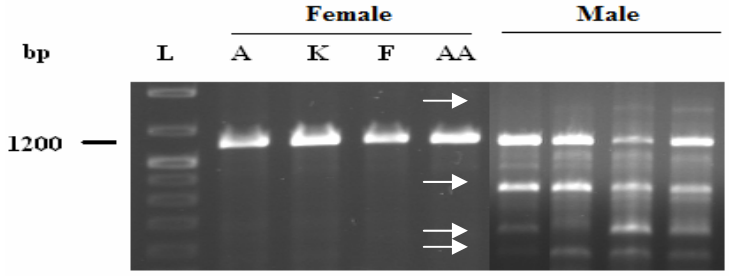

Figure 1: Identification of male and female individual plants of Pistacia cultivars by FPKI016 primer. L: DNA ladder, A: Akbary, K: Kalehghochi, F: Fandoghi, AA: Akbar Aghaii

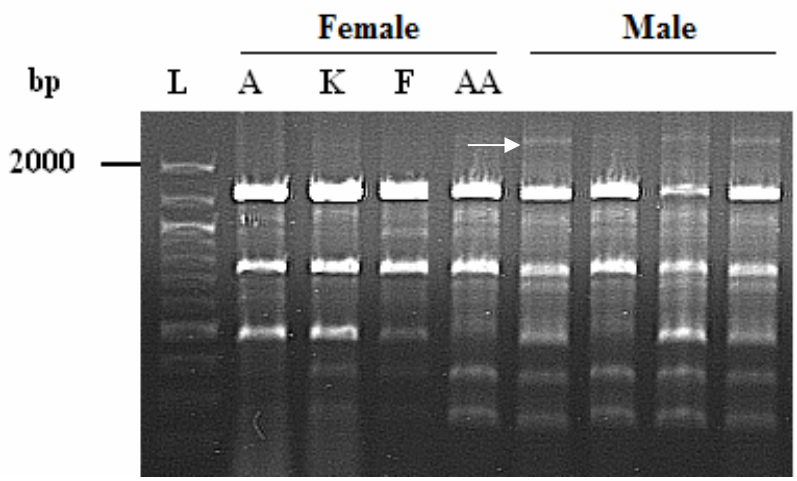

Figure 2: Identification of male and female individual plants of Pistacia cultivars by FPK105 primer. L: DNA ladder, A: Akbary, K: Kalehghochi, F: Fandoghi, AA: Akbar Aghaii

Discussion

Efforts to identify dioecious plants sex type in an early stage of development is important for selecting female and male pistacia plants before transfer to the field in order to save time and reduce costs. To date, several molecular markers for sex type discrimination in dioecious plants, including Pistacia (Hormaza et al., 1994) and papaya (Urasaki et al., 2002) have been reported. In the present study, from the reported primer (OP008) by Hormaza et al., (1994), we tried to discriminate sex type in four Pistacia cultivars, but could not distinguish between male and female plants under several PCR amplification conditions (data not shown). Based on these unsatisfactory results we concluded that new markers are required to distinguish sex type of desirable Pistacia cultivars. In order to find molecular differences between male and female plants, different attempt such as immunochemistry, differences in proteins and enzymes, comparison of mRNA and tRNA populations or RNA hybridization kinetics, have been made in several dioecious species (Durand and Durand, 1990). These studies deal with gene expression and could just show a differential expression of common genes between males and females. A better approach to understand how sexual determination operates in dioecious species is to study the differences at the DNA level. The discovery of markers linked to sex determining genes could eventually allow us to clone the gene(s) involved in this process. Although promising results have been obtained with *Asparagus* (Jamsar et al., 2004) using RFLPs (Bracale et al., 1991), the RAPD technique appears to offer better prospects for rapid progress. Its advantages relative to RFLPs include technical simplicity, lower cost per data unit, small amount of DNA required and the higher level of polymorphism obtained with RAPDs (Waugh and Powell, 1992; Williams et al., 1993). Among 32 primers used in this study only 2 primers could reveal sex differentiation in pistacia cultivars. It has been proposed that the basic scheme of sex determination in animals involves a key gene that activates a cascade of regulatory genes (Truong et al., 1991). In plants, the system could be similar. If this is the case, of several to many genes could be involved in the differentiation of male and female flowers in dioecious plants, but sex determination could be controlled by a single locus acting as a trigger. In such a scenario, genes having the genetic information for carpels or stamens development would be present in both male and female plants with one major gene being the only difference between the two sexes (Irish and Nelson, 1989). Durand and Durand (1990) reported that a single major gene controls sex determination in some plant genera (e.g. *Asparagus* and *Vitis),* although there are exceptions like *Mercurialis* where the system is more complex with three genes involved in sex determination. Pistachio may have a similar system with a single major gene controlling sex determination Here we propose that, we may have identified a marker which possibly which is closely linked to a sex-determining chromosome segment in *P. vera* and this marker can be used to screen sex of pistacia seedlings well before they attain reproductive stage.

Acknowledgment

Authors thanks the Research Council of the University of Isfahan for their support of research project No. 860411.

References

1- Bawa K. S. (1980) Evolution of dioecy in flowering plants. Annual Review of Ecology and Systematic 11:15-39.

2- Bracale M., Caporali E., Galli M. G., Longo C., Marziani-Longo G., Rossi G., Spada A., Soave C., Falavigna A., Rafaldi F., Maestri E., Restivo F. M. and Tassi F. (1991) Sex determination and differentiation in *Asparagus officinalis* L. Plant Science 80:67-77.

3- Doyle J. J., and Doyle J. L. (1987) A rapid isolation procedure for small quantities of fresh leaf tissue. Phytochemistry Bulletin 19:11-15.

4- Durand R. and Durand B. (1990) Sexual determination and sexual differentiation. Critical Review of Plant Science 9:295-316.

5- Hormaza J. I., Dollo L. and Polito V. S. (1994) Identification of a RAPD marker linked to sex determination in *Pistacia vera* using bulked sergeant analysis. Theoretical and Applied Genetic 89: 9-13.

6- Irish E. E. and Nelson T. (1989) Sex determination in monoecious and dioecious plants. Plant Cell 1: 737-744.

7- Jamsari A., Nitz I., Reamon S. M. and Jung C. (2004) BAC-derived diagnostic markers for sex determination in asparagus. Theoretical and Applied. Genetic 108: 1140–1146.

8- Lewis K. R., and John B. (1968) The chromosomal basis of sex determination. International Review of Cytology 23: 277-379.

9- Lynch M. and Milligan B. G. (1994) Analysis of population genetic structure within RAPD markers. Mol. Ecol. 3: 91-99.

10- Lynch M. and Milligan B. G. (1994) Analysis of population genetic structure within RAPD markers. Molecular Ecology 3: 91-99.

11- Mulcahy D. L., Cresti M., Linskens H. F., Intrieri C., Silverstoni, Vignani O. R. and Pancaldi M. (1995) DNA fingerprinting of Italian grape varieties: a test of reliability in RAPDs. Advance in Horticulture Science 9: 185-187.

12- Mulcahy D. L., Cresti M., Sansavini S., Douglas G. C., Linskens H. F., Bergamini G., Vignani R. and Pancaldi M. (1993) The use of random amplified polymorphic DNAs to fingerprint apple genotypes. Science Horticulture 54: 89-96.

13- Parker J. S. and Clark M. S. (1991) Dosage chromosome systems in plants. Plant Science 80:79-92.

14- Truong A., Heinderyckx T., Install M. and Negrutiu P. (1991) Sex determination and differentiation in organisms other than higher plants. Plant Science 80:201-211.

15- Urasaki N., Tokumoto M. Tarora K., Ban Y., Kayano T., Tanaka H., Oku H., Chinen I. and Terauchi R. (2002) A male and hermaphrodite specific RAPD marker for papaya (Carica papaya L.) Theoretical. Applied Genetic 104: 281–285.

16- Wannan B. S. and Quinn C. J. (1991) Floral structure and evolution in the Anacardiaceae. Botany. Journal of Linneh Society 107:349-385.

17- Waugh R. and Powell W. (1992) Using RAPD markers for crop improvement. Biotechnology 10:186-191

18- Westergaard M. (1958) The mechanism of sex determination in dioecious flowering plants. Advance Genetic 9:217-281.

19- Williams J., Anne G., Kubelik K., Kenneth R., Livak J., Antoni R., Scott J. and Tingey V. (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Research 18(22): 6531-6535.

20- Williams J. G. K., Hanafey M. K., Rafalski J. A. and Tingey S. V. (1993) Genetic analysis using random amplified polymorphic DNA markers. Method in Enzymology 218:704-740.