AES/CE MAR 84

Workgroup/Department: Pistachio/Pomology

University of California
Division of Agricultural Sciences

Final PROGRESS REPORT

Pistachio Nursery Group

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Project Year 2001 Anticipated Duration of Project 1 year (completed)

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Project Title Part 1. Establishment of molecular markers to determine genetic variation in pistachio rootstocks.

Objectives:

The ultimate objective of this proposal is to develop molecular markers to disclose the origin of observed genetic variation among the pistachio rootstocks "UCB1".

Summary Results

Microsatellite markers have been developed to investigate the variation among the UCB1 rootstocks. Molecular markers such as microsatellites and SRAP markers revealed that the variation among the UCB1s is of genetic origin and this variation is due to the heterozygosity of the parental genotypes.

Development of SSR markers

To develop microsatellite markers an enrichment library has been constructed following the protocol of Hamilton et al (1999) with some modifications. Genomic DNA was extracted from leaf tissue of pistachio cultivar 'Kerman'by Qiagen maxi prep according to the manufacturer's protocol. Approximately 15 µg of DNA was digested with restriction enzyme Rsa1 and Nhe1 which resulted in DNA fragments at the range of 300-800bp. These DNA fragments were cleaned with Qia Quick DNA purification column (50ul final elution) and dephosphorylated with Calf intestinal phosphatase (CIP) at 37°C for two hours and re-cleaned with Qiaquick DNA column (Qiagen) with 30ul final elution.

To generate an adaptor, SNX F and SNX R (Phosphorylated) was hybridized in a heating block set at 80°C and cooled slowly over 1 hour. Ligation of 99 pmol double-stranded adaptors to 33 pmol blunt ended DNA fragments was done in the presence of restriction enzyme Xmn1 that cuts any SNX linker dimmers. PCR was performed on the linker ligated DNA and run on 1.5% agarose gel. A smear at the range of 300-800 bp showed success in the ligation process.
Linker ligated DNA was hybridized to biotinylated repeat oligo (CA) n and (CT)n. 50µl of streptaviodin coated magnetic beads were prepared according to the manufacture protocol and biotinylated hybridized

DNA fragments were added to it. After incubation at 43°C for several hours, magnetic beads were washed twice with 200µl, 2xSSC, 3times with 200µl 1xSSC and 3times with 200µl with 0.5 µl 0.5xSSC. Final wash was performed by shifting magnetic beads to a new tube and washing with 200µl 0.5xSSC solution. Each incubation was for five minutes at 48°C. A high power magnetic stand was used each time to separate magnetic beads and washing solution. After final washing, 30µl low TE buffer was added to magnetic beads and incubated at 98°C for 15 minutes, then quickly centrifuged for a few seconds, put in the magnetic stand and when the magnetic beads were out of the way, TE buffer was removed which became elution 1. The procedure was repeated with 60 µl of TE buffer, which became elution 2. Next 30 µl TE buffer was added to the magnetic beads and stored at -4°C for future use. PCR was conducted on the enriched DNA by taking different dilution. A light smear at the range of 300-800 bp on 1.5% agarose gel showed success in enrichment procedure.

Amplified enriched DNA was cleaned with Qia quick DNA purification kit (Qiagen).To get more efficient transformation with the TA cloning kit (Invitrogen) , 1µl taq DNA polymerase ,with dATP , 10x PCR buffer was added to the enriched DNA , incubated for 10 minutes and directly added to the vector pCR2.1 Toppo cloning vector (Invitrogen, Carlsbad, CA, USA) and Ecoli competent cells (Top 10, Invitrogen) according to the manufacturer's protocol To check the insert size, white colonies were transferred to tubes containing 20ul of 10mM Tris -HCl pH 8.5 and incubated for 10min at 95°C, and 0.5 µl was used as a template in PCR amplification with two vector primers. At the same time colonies were transferred to LB Kanamycin freezing medium. Plasmids were isolated and sequencing were performed by using M13 (-21)F primer. In some cases when the insert was long, re-sequencing was done by using M13R primer. For each unique microsatellite from both the enriched and non enriched libraries that contained sufficient reliable flanking sequence, PCR primers were selected using the primer selection computer program Primer 5.0 (Lander ,Cambridge, Mass,USA). The forward primers were labeled with IR800 to be detected on an automated DNA sequencer (LiCor).

PCR was carried out under the following conditions: 100-150 ng of template DNA, 250 nM of each primer, 200 µM of dNTPs, 1 U of Taq Polymerase, 1.5-2 mM of MgCl 2 . The reaction, depending on the primer pair, was run for 35-45 cycles (denaturing at 94°C for 1 min, annealing at 55°C or 60°C for 1 min, with a 2-min extension at 72°C), followed by a single extension at 72°C for 60 min. The amplification products were detected on 6 % polyacrylamide gels using a Li-Cor DNA sequencer.

Results

Microsatellite marker

The SSR markers developed showed high efficiency for detecting the genetic variation among the UCB1. Eight SSR markers have been developed for this project (Table 1).

Table 1: Polymorphic SSR markers for pistachio, motifs and their expected fragment size.

Name	Motifs	Fragment size (bp)
PIMS16	$(CT)_{31}$	190
PIMS22	$(GT)_{20}$	237
CMS7	$(CT)_{16}$	150
CMS47N	$(GT)_{15}$	193
PIMS31	$(GT)_{20}$	146
PIMS33	$(GT)_{8}$	182
PIMS42	$(CA)_{45}$	204
PIMS45	$(GT)_{18}$	224

Genetic variation among "UCB1"

The rootstock "UCB1" has been generated by hybridization of two pistachio species, *Pistacia atlantica x Pistacia integerrima*. We assumed that the variation among 'UCB1` was because of the heterozygosity of the parents, which resulted in F1s with different allelic constitutions.

We investigated leaf samples of six "UCB1" roostock selections. One sample from UC Davis (Louise Ferguson) and 5 from S&J Ranch. We also received leaf samples of *P. atlantica*, *P. intergerrima* from UC Davis, S&J Ranch and Kevin Olson.

We tested two samples of *P. altlantica* and two samples of *P. intergerrima* using "Sequence-based (related) amplified polymorphism" molecular markers (SRAP marker). SB marker could reveal allelic differences among the samples of *P. atlantica* and *P. intergerrima* a well as among the 6 UCB1 rootstocks (Fig. 1).