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MALE SPECIFIC GENE EXPRESSION IN DIOECIOUS *PHOENIX DACTYLIFERA* (DATE PALM) TREE AT FLOWERING STAGE

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Abstract

Date palm is a long-living and evergreen important tree in the semiarid regions. Its fruit is rich in carbohydrate and fibres. Transcriptional profiling was compared among male and female trees of dioecious date palm at flowering stage. Male specific genes are expressed at flowering stage which was studied using the cDNA–SCoT marker. We developed sequence characterized amplified region (SCAR) markers of size 253 bp from male tree based on cDNA-SCoT fingerprinting. Further, developed SCAR marker was validated on the independently collected samples of both types of trees at flowering stage. The unique and specific band (253 bp) was amplified from male samples only whereas it was absent from female samples.

Key words: SCAR, cDNA, SCoT, Transcriptional profiling, Sequencing.

Introduction

Date Palm (*Phoenix dactylifera* L.) (family: Arecaceae) is a main crop of arid region of west Asia and North Africa. During the past three centuries, dates were also introduced to other countries in South America, Mexico, India/Pakistan, southern Africa, Australia, and United States. It is the most important species in the Palm family, which comprises about 200 genera and more than 2,500 species (El Hadrami & El Hadrami, 2009; Jain *et al.,* 2011). The number of date palm trees is estimated as 25 million in the Kingdom of Saudi Arabia (Al-Abbad *et al.*, 2011) and more than 450 different date varieties are found across the country. The Kingdom produces nearly a million tons of dates annually accounting for about 15% of the global date production (Al-Abdoulhadi, *et al.,* 2011).

Date palm genetic material is highly diversified, since a lot of cultivated varieties have been described. Date palm is dioecious plant i.e., male and female flowers are produced on separate tree. Morphological markers are also used for cultivar identification, however these markers overlap to each other at pre-flowering stage and make more complex for date palm taxonomist. Various molecular markers have been developed for sex determination in date palm **(**Elmeer and Mattat, 2012; Dhawan *et al.,* 2013; Al-Mahmoud *et al.,* 2012)**.** However, gender identification is a cumbersome process for male plant because mostly they are seed borne and are hardly identical to any female cultivar (Al-Khalifah, *et al.,* 2012).

There are around 6% angiosperms in the world which are dioecious bearing male and female flowers on separate plants (Renner & Ricklefs, 1995; Charlesworth, 2002). Different genes are expressed in male and female plants at flowering stage (Chawla *et al.,* 2015). Date palm is slow growing plant; the gender of date palm cannot be differentiated at early stage of seedling. It can be done at flowering stage which take around 5-6 years. Sex determination in dioecious plant is based on regulation of X/Y chromosome (Charlesworth, 2002). Difference in male and female plants can be seen in reproductive organs which can be occur through repression or abortion of sex organs in unisexual flower and differential growth (Chuck, 2010; Matsunaga, 2006).

Several molecular markers have been used for the assessment of genetic diversity such as random amplified polymorphic DNA (RAPD), inter simple sequence repeat (ISSR), amplified fragment length polymorphisms (AFLP), restriction fragment length polymorphisms (RFLP) and simple sequence repeat (SSR) (Sedra *et al.,*1998; Abdallah *et al.,* 2000; Trifi, *et al.,* 2000; Al-Khalifah & Askari, 2003; Zehdi *et al.,* 2002; Rhouma *et al.,* 2007; Corniquel and Mercier, 1994; Zehdi *et al.,* 2004). Recently, new marker has been developed for the assessment of genetic diversity, called Start Codon Targeted (SCoT) marker (Collard & Mackill, 2009). This marker is more reproducible than the RAPD as it has longer primer sequence. Start codon (ATG) and flanking sequences are highly conserved in the plant genes (Sawant *et al*., 1999). In SCoT marker profiling, single primer is used as like in ISSR and RAPD markers. The SCoT marker has been used for the assessment of genetic diversity in Saudi Arabian date palm cultivars and gave reprodicible results among the cultivars (Al-Qurainy *et al.,* 2015). However, SCoT markers can be developed from the transcribed regions and might be linked to the gene function as studied in sugarcane (*Mangifera indica* L.) (Wu *et al*., 2013; Luo *et al*., 2014) etc. In the present study, we performed cDNA-SCoT fingerprint for the study of specific gene expression in dioceious date palm trees at flowering stage which has not been done till now.

Material and Methods

Sample collection: Date palm male and female flowers were collected in liquid nitrogen from Agricultural Research Station, Dirab, Riyadh, in the month of February 2015 (Table 1). Female flowers were collected before pollination to avoid the pollens. After collection, all samples were kept in -80ºC till experiment designing.

Table 1. List of date palm cultivars and male trees.

S.No.	Plants	Accession number
1.	Male-1	M1
2.	Male-2	M2
3.	Male-3	M3
4.	Male-4	M4
5.	Male-5	M5
6.	Male-6	MC1 (male date palm for validation of SCAR marker)
7.	Male-7	MC2 (male date palm for validation of SCAR marker)
8.	Barhi	F1
9.	Khalas	F2
10.	Sukkari	F3
11.	Ruthana	F4
12.	Ruzeiz	F5
13.	Wesaily	FC1 (female date palm for validation of SCAR marker)
14.	Sullaj	FC2 (female date palm for validation of SCAR marker)

Total RNA isolation and cDNA preparation: Total RNA was isolated from the male and female flowers (Table 1) using the RNeasy Plant mini kit (Qiagen) following the instructions given in the manual. The quality and quantity were measured with Nanodrop 8000 spectrophotometer (Thermo Scientific). cDNA was prepared using the QuantiTect Reverse Transcription Kit (Qiagen) for cDNA-SCoT marker profiling of male and female flowers. Further, cDNA was purified using the PCR purification Kit (Qiagen).

SCoT marker profiling: PCR reaction was performed in a total volume of 25 µl using the SCoT primers (Table 2) for SCoT marker profiling. The reaction mixture (PCR bead purchased from the GE health care) contained all components except primer and template DNA. Double distilled water was added in the tube contained reaction mixture followed by primer (20 picomole) and template cDNA (50 ng). The SCoT primers were selected from the literature for monocot plant species (Collard & Mackill, 2009). PCR reaction was performed in AB Veriti 96 well Thermalcycler. The cycling profile was: 94ºC for 3 min, 45 cycles at 94ºC 1min, 44.5ºC 30s, 72ºC 1 min and a cycle of 72ºC for 5 min. The amplified products were resolved on 1.2% TBE agarose gel.

Table 2. List of SCoT primer sequences (Collard and Mackill, 2009).

S.No.	Primer code	Primer sequence
1.	SCoT-1	5′-CAACAATGGCTACCACCA-3′
2.	SCoT-2	5′-CAACAATGGCTACCACCC-3′
3.	SCoT-3	5′-CAACAATGGCTACCACCG-3′
4.	SCoT-4	5′-CAACAATGGCTACCACCT-3′
5.	SCoT-5	5′-CAACAATGGCTACCACGC-3′
6.	SCoT-6	5′-CAACAATGGCTACCACGG-3′
7.	SCoT-7	5′-CAACAATGGCTACCACGT-3′
8.	SCoT-8	5′-CAACAATGGCTACCAGCA-3′
9.	SCoT-9	5′-CAACAATGGCTACCAGCC-3′
10.	SCoT-10	5′-AAGCAATGGCTACCACCA-3′

Selection of unique amplicon: The putative marker amplified by the SCoT primer was excised from 1.2% agarose gel with sterile gel slicer. The excised band was purified using Wizard SV Gel system kit (Promega). The purified PCR products were sequenced at Macrogen, Inc., Korea using the same SCoT primer as used for amplification. After sequencing, all SCoT fragment sequences were aligned using CLUSTALX software (Thompson *et al.,* 1997).

Designing of new primer based on cDNA-SCoT amplicon sequence: The obtained sequences were BLAST at GenBank database to confirm our sequence (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Based on the characterized sequence, new primers were designed named (SCARF: 5′- CGGCACTACGGCTTCAGGAC-3′ and SCARR: 5′- TCCCGAAGTGGTTGAAGG -3′) for the development of SCAR marker. There was no potential dimers and hairpin formation found within these primers and it was confirmed using the Oligo 5.0 software.

Validation of designed SCAR primer: DNA sequence was submitted to the GenBank database after homology search and alignment. Homology searches were performed within GenBank's nonredundant database using the BLAST 2.2.8 (Basic Local Alignment Search Tool) algorithm at http://blast.ncbi.nlm.nih.gov/Blast.cgi of the National Center for Biotechnology Information (NCBI), with the program BLASTX. The developed SCAR marker was validated on other date palm samples collected at flowering stage. The PCR reaction was performed in a 25 µl volume in AB Veriti 96 well Thermalcycler. The PCR program had 94ºC for 3 min, 36 cycles at 94ºC 1 min, 51.0ºC 30s, 72ºC 1 min and a cycle of 72ºC for 5 min. The amplified products were resolved on 1.2% TBE agarose gel.

Results and Discussion

In flowering plants, the transition depends on a complex genetic network that integrates information from environmental and endogenous cues (Amasino, 2010). External and Internal factors also regulate whether a meristem produces vegetative or reproductive structures (Gilbert, 2000). Differential gene expression is different in plant species at different development stages (Chawla *et al*., 2015) and it also vary under biotic and abiotic stresses (Jiang *et al.,* 2012; Lu *et al.,* 2014). We studied gene expression in male and female date palm trees at flowering stage using the cDNA-SCoT fingerprinting. Some unique bands were amplified from the male plant samples, while absent from the female plant samples (Fig. 1).

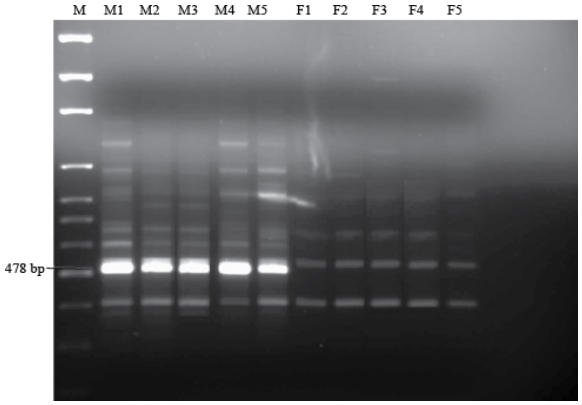

Fig. 1. cDNA-SCoT marker profiling using SCoT primer-2. M: 1 kb ladder.

We used ten primers for cDNA-SCoT marker profiling among the male and female trees. The unique band was amplified in male trees (size: 478 bp) with SCoT primer-3 (5′-CAACAATGGCTACCACCG-3′), whereas it was not amplified in female trees. This male specific band was cloned and sequenced at Macrogen Inc., Korea (Fig. 2). The sequence was submitted to the Genbank database to access publically (KU243700). Similarly, cDNA-SCoT marker has been used for differential gene expression in sugarcane (Wu *et al*., 2013). Oligo dT cDNA-SCoT technique was used for differential gene expression analysis under salinity stress in *Mangifera indica* (Luo *et al*., 2014). In dioecious liverwort *Pellia endiviifolia* female-specific genes are expressed which is connected to the archegonia production (Sierocka *et al*., 2014). The SCoT and RAPD markers were also employed for gender identification of Egyptian date palm trees (Adawy *et al.,* 2014) at seedling stage.

The sequence was BLAST for homology search at NCBI database (http://www.ncbi.nlm.nih.gov/nucleotide) and it showed the sequence similarity to the pollen grains of *Elaeis guineensis* Jacq (Family: Arecaceae). The predicted amino acid sequence showed the homology to the deduced amino acid sequences of the pectate lyase gene expressed in the pollen grains (Fig. 3). The pectate lyase gene is specifically expressed during the microsporogenesis in tomato (Rogers *et al.,* 1992). The designed new SCAR primers were used for the amplification of cDNA of both male and female date palm samples, the unique band was appeared in male plant samples of size 253 bp whereas it was absent in female plant samples. Further, the designed SCAR marker was validated on independently collected samples (MC1, MC2; male); and (FC1, FC2; female) from which total transcripts were extracted from flowers (Fig. 4) and found same results as obtained from the samples used in the development of SCAR marker.

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                ....|....| ....|....| ....|....| ....|....| ....|....|
                         10         20         30         40         50
SCoT_Male       ACGTCCACAT CGCCTTCGGC TGCCAGATAA GCATCCAGTT CGTGCAAAAC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                         60         70         80         90        100
SCoT_Male       GTCATCATCC ACGGCCTCCA CATCCATGAC ATCAAACCCG GGAACGGCGG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        110        120        130        140        150
SCoT_Male       CATGATCAGG GACTCCCTCC GGCACTACGG CTTCAGGACC AAGAGCGATG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        160        170        180        190        200
SCoT_Male       GCGATGGCAT CTCCATCTAC GGCTCCAGCG ACATCTGGAT CGACCACTGC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        210        220        230        240        250
SCoT_Male       TCCATGAGGA ACTGTGCTGA CGGCCTCATC GATGCCATCG AGGCATCCAC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        260        270        280        290        300
SCoT_Male       TGCCATCACC ATTTCCAACT GCCACTTCGC CCGCCACAAC GACGTGCTGC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        310        320        330        340        350
SCoT_Male       TGTTCGGTGC CAGCGATTCG AACGAGAGGG ACTCGATAAT GCAGGTCACT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        360        370        380        390        400
SCoT_Male       GTTGCCTTCA ACCACTTCGG GAAAGGCCTT GTGCAGAGGA TGCCGAGGTG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        410        420        430        440        450
SCoT_Male       CCGCTGGGGC TTCTTCCACG TCGTCAACAA TGACTACACC CACTGGATGA

                ....|....| ....|....| ....|...
                        460        470
SCoT_Male       TGTATGCCAT CGGTGGTAGC CATTGTTG
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Fig. 2. Male date palm specific sequence generated in cDNA-SCoT profiling.

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                ....|....| ....|....| ....|....| ....|....| ....|....|
                         10         20         30         40         50
Protein_Se      VHIAFGCQIS IQFVQNVIIH GLHIHDIKPG NGGMIRDSLR HYGFRTKSDG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                         60         70         80         90        100
Protein_Se      DGISIYGSSD IWIDHCSMRN CADGLIDAIE ASTAITISNC HFARHNDVLL


                ....|....| ....|....| ....|....| ....|....| ....|....|
                        110        120        130        140        150
Protein_Se      FGASDSNERD SIMQVTVAFN HFGKGLVQRM PRCRWGFFHV VNNDYTHWMM

                ....|...

Protein_Se      YAIGGSHC
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Fig. 3. Predicted protein (pectate lyase) from the cDNA-SCoT marker fingerprinting.

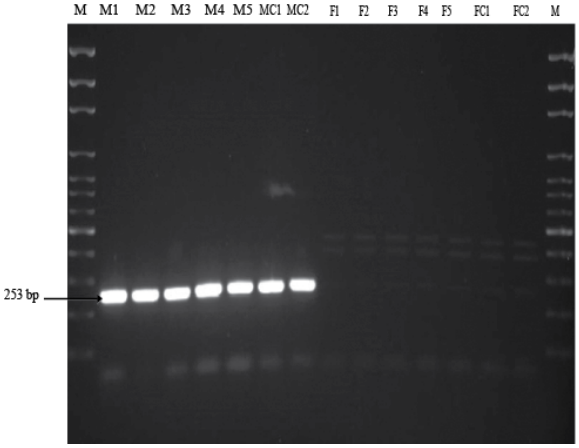

Fig. 4. SCAR marker based on cDNA-SCoT marker profiling. M: 1 kb ladder.

In conclusion, the applied cDNA-SCoT marker gave clear gene expression difference in dioecious date palm at flowering stage. The marker is simple, inexpensive and more reproducible. The cDNA-SCoT marker could be used for the study of differential gene expression and their identification. Further, discovered new genes using the cDNA-SCoT marker, could be used in breeding program for crop improvement.

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