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Short Communication

In silico development and validation of EST derived new SSR markers for drought tolerance in *Cicer arietinum* L.

Juhi Bhardwaj[1,2], Nilima Kumari[2], Rebecca Ford[3], Renu Yadav[1,5], Insoo Choi[4] and Rajendra Kumar[1,6,*]

[1]Department of Biotechnology, Sardar Vallabhbhai Patel University of Agriculture & Technology, Meerut 250 110; [2]Department of Biosciences and Biotechnology, Banasthali Vidyapeeth, Rajasthan; [3]Department of Agriculture and Food Systems, Melbourne School of Land and Environment, The University of Melbourne, Victoria, Australia 3010; [4]Department of Plant Bioscience, Pusan National University, Miryang, Korea; [5]Department of Biotechnology, B.C.M.&T., C.C.S.University, Meerut 250 110; [6]Division of Genetics, Indian Agricultural Research Institute, New Delhi 110 012

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Abstract

Microsatellites have become a marker of choice in plant genetics and breeding research due to their unilocus, multi-allelic and highly reproducible nature. In present study EST sequences isolated from chickpea root under water stress were assessed for the frequency, type and distribution of SSR motifs. Out of the 6400 ESTs (accession no. CDO 38847 to GR 394575), SSR sequences were identified in 348 singleton contigs and from these, 50 flanking primer pairs were designed. Out of these, 41 amplified a marker of the expected size range across nineteen diverse chickpea accessions. 11 of the primer pairs were size polymorphic with an average PIC value of 0.59 across the nineteen genotypes assessed.

Key words: Chickpea, EST, microsatellites, drought tolerance

Chickpea is the world's third most important food legume [1]. Drought and salinity are the two most important abiotic stresses that alter plant water status and severely limit plant growth and development [2]. For future targeted breeding programs, the locations of the putative candidate genes imparting tolerance must be identified through molecular mapping. Microsatellite markers are commonly used for genotyping for diversity and genome mapping studies [3-7]. Although several libraries of SSR markers exist for chickpea (2), but EST-SSR markers are required to aid in assessment of broader diversity, producing denser genome maps with potential functional association. Many expressed sequence tag (EST) libraries have generated vast amounts of publicly available sequence data from plant species including chickpea (2, 8, 9, 10, 11). *In silico* data mining of the existing EST clone libraries provide an alternate approach for the isolation and identification of novel SSRs. The present study aimed to (i) mine the existing publically available chickpea EST libraries for SSR and characterize the identified EST-SSR; (ii) design flanking primers to amplify the EST-SSR; and (iii) validate the amplification of the new EST-SSR sequences on chickpea drought tolerant/resistant genotypes.

A total of 6400 chickpea EST sequences available in the NCBI database (http://www.ncbi.nlm.nih.gov/) were downloaded (Accession No. CDO 38847-GR 394575). These were clustered into contigs and singletons using CAP 3 software (http://deepc2.psi.iastate.edu/aat/sas.html). The resultant 348 contigs were mined for novel SSRs using tandem repeat occurrence locator (TROLL) (http://purl.oclc.org/NET/websat). Primer pairs were designed using Primer3 software (Rozen and Skaletsky, 1997;

*Corresponding author's e-mail: rajendrak64@yahoo.co.in
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Table 1. List of chickpea accessions used in the study

S.N.	Accessions	Source/origin
1.	Pusa 1088	I.A.R.I., New Delhi, India
2.	BG 112	I.A.R.I., New Delhi, India
3.	ILC 3279	ICARDA, Syria
4.	ICC 4958	ICRISAT, India
5.	BG 1053	I.A.R.I., New Delhi, India
6.	Pusa 256	I.A.R.I., New Delhi, India
7.	Pusa 1108	I.A.R.I., New Delhi, India
8.	Pusa 1005	I.A.R.I., New Delhi, India
9.	BG 1103	I.A.R.I., New Delhi, India
10.	BG 2004	I.A.R.I., New Delhi
11.	BG 1092	I.A.R.I., New Delhi, India
12.	JG 74	J.N.K.V.V,M.P.,India
13.	ICCV 2	ICRISAT, India
14.	Pusa 1105	I.A.R.I., New Delhi, India
15.	K850	CSAUA&T, U.P., India
16.	BG 72	I.A.R.I., New Delhi, India
17.	JG 11	J.N.K.V.V., M.P., India
18.	BG 1073	I.A.R.I., New Delhi, India
19.	BG 1063	I.A.R.I., New Delhi, India

http://fokker.wi.mit.edu/primer3) with the following optimal parameters: 100-280 bp product length; 40-80% GC content. 50-60^{o}C Tm; and a primer length of 15-25 bases. Primers were synthesized by Imperial Life Sciences, USA and designated as SVP (Sardar Vallabhbhai Patel) with a numerical identification.

DNA was isolated from fresh, young leaf tissues of 19 chickpea accesions (Table 1) using the CTAB method [12]. All of the accessions are drought tolerant and originated from diverse geographical/genetic sources (Table 1). Genomic DNA quality and quantity was estimated by agarose gel electrophoresis. PCR amplifications were carried out in 10 µl reaction volumes containing 25ng of genomic DNA, PCR buffer (1X) (Bangalore Genei, India), 0.5 µM (Integrated DNA Technology, Imperial Life Sciences, USA) of each primer, 125mM of each dNTP (Bangalore Genei, India), 1mM MgCl2 (Bangalore Genei, India) and 1U of *Taq* DNA polymerase (Bangalore Genei, India). The following touchdown amplification profile was used: Initial denaturation at 95°C for 2 min followed by 35 cycles of 95°C for 20 s (denaturation), 54-60°C for 50 s (annealing), 72°C for 50 s (extension) followed by a final extension of 72°C for 7 min. The amplification products were separated on agarose gel and size were

Table 2. List of polymorphic EST and SSR markers

S.No.	Primers	Primer sequence (5'-----3')	Repeat motif	PIC
1.	SVP 162	F TCTCAGTTCCCTCATTCAAC R ATTTCTCCCACCAGTCTTTT	(TTC) 5	0.85
2.	SVP 180	F CCCCAAACAACATTATCC R GAACTGGTGGGAAATACAC	(ACAA) 4	0.68
3.	SVP 215	F TCTCCCAAATTCTTGTCCCA R ACACAGGTAGTGGTGGTGGC	(CCA) 4	0.11
4.	SVP 219	F CAAAATCCCACACCACTACT R GTTTCCATAACCACCTGTGT	(TGG) 5	0.98
5.	SVP 221	F GTAGGACCGATGAATATGGA R CTTATTTTACCGACTGCACC	(GGACA) 2	0.91
6.	SVP 309	F ATATGAGCAACACCTTCTGC R CTACTTTTACGTGGAGCGTT	(ATG) 4	0.20
7.	SVP 311	F GGGTCCAATAATCTGTTGTG R AGCCACATAAACATGGAGA	(CCA) 5	0.68
8.	SVP 31	F CTTTTGGCTCCACCCATT RGAGGAAAGGTTATTCGGGTAAA	(G) 10	0.56
9.	SVP 11	F TCTCAGTTCCCTCATTCAAC R ATTTCTCCCACCAGTCTTTT	(AAC) 5 (AATTC)	30.65
10.	SVP 181	F ATATGCAGCCAGCAAAACTG R GGTTCGGATTGTCACTTGCT	(G) 11	0.56
11.	SVP 64	F GCAAGCACACCAGCTTTGTA R TGGCAATGACAAGATCTCCA	(ATGTT) 3	0.37

estimated alongside a 100 bp DNA ladder (Bangalore Genei, India).

Functional annotation of newly designed markers was obtained from Gen Bank using the blast X algorithm against the nr database [13]. The Contigs used for markers development, were translated using TranSeq (http://www.ebi.ac.uk/Tools/emboss/transeq/index. html) and were submitted in the AmiGO term browser (http://amigo.geneontology.org/cgibin/amigo/search.cgi) to find its molecular function. The Pfam database was also used to infer gene function (http://pfam.janelia.org/).

The use of CAP3 software aided in identification of overlapping sequences among ESTs and generation of consensus contiguous sequences. Out of the 6400 ESTs assessed, 18.4% fell into shared and contiguous sequences (1,178) indicating a relatively high level of redundancy within and between the chickpea EST databases.

Within the EST-derived contigs the most common repeats were CT, TA (di), AGA, TCA,TGG (tri), CCAC, ANTC (Tetra), AAANA, TCTCN (penta), and AATATT (hexa); which varied in length from 2 to 10 units. In total, 348 of the 1,778 contigs contained 19.6 % SSRs, of which 50 possessed sufficient flanking sequences to design primer pairs. Out of the 50 SSR primer pairs designed in the current study, 41 amplified a product in the expected size range in each of the nineteen chickpea accessions assessed (Table1) and 11 of these produced a size polymorphism with an average PIC value of 0.57 across the nineteen genotypes (Table 2). In future, the SSR developed from drought-associated EST could be be mapped to identify potential candidate drought tolerance genes and their functional association with tolerance to water stress.

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