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An attempt to reveal Ty3-*gypsy* retrotransposon-based S-SAP markers in chickpea

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ABSTRACT

Retrotransposons are present in high copy number in plant genomes. They show a considerable degree of sequence heterogeneity and insertional polymorphism, both within and between species. The retrotransposon based genetic diversity analysis technique Sequence-Specific Amplification Polymorphism (S-SAP) has recently been derived from the Amplified Fragment Length Polymorphism (AFLP) technique. It produces amplified fragments containing a retrotransposon LTR sequence at one end and a host restriction site at the other. This is the first attempt to develop *gypsy* retrotransposon-based S-SAP markers in chickpea. We tried to find possible S-SAP markers in 30 chickpea (*Cicer arietinum* L.) accessions widely grown around the world. Polymorphisms were detected in eight accessions, using PCR primers designed from the long terminal repeat (LTR) of the chickpea retrotransposon *CARE*-1 and a set of three selective MseI+3 primers. From the 215 bands scored, 12 were polymorphic.

KEYWORDS

Chickpea;
Retrotransposon;
Molecular marker;
S-SAP.

INTRODUCTION

Plant genomes are dominated by retroelements[6,33]. On the basis of presence or absence of long terminal repeats (LTRs) at their termini these elements have been classified into two groups- LTR retrotransposons and non-LTR retrotransposons. LTR retrotransposons are further divided into Ty1-*copia* and Ty3-*gypsy* subgroups. In Ty1-*copia* elements the integrase domain is present between protease and reverse transcriptase domain while in Ty3-*gypsy* elements, it is located between ribonuclease H domain and 3'LTR region[2]. Retrotransposons show abundant variation in copy number and genomic localization among even closely related plant species[4,18,26]. The dispersion, ubiquity, sequence heterogeneity and insertional polymorphism of retrotransposons, both within and between plant species provide an excellent basis for the development of marker systems. Several retrotransposon-based molecular marker techniques have emerged during the last few years[5,7,11,34]. One such molecular marker technique is Sequence-Specific Amplification Polymorphism (S-SAP). S-SAP is a multiplex amplified fragment length polymorphism (AFLP) like technique that displays individual retrotransposon insertion as bands on a sequencing gel. Fragments are amplified by PCR, using

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one primer from the LTR terminus of the retrotransposon and other from the nearest restriction endonuclease site (Figure 1)[34].

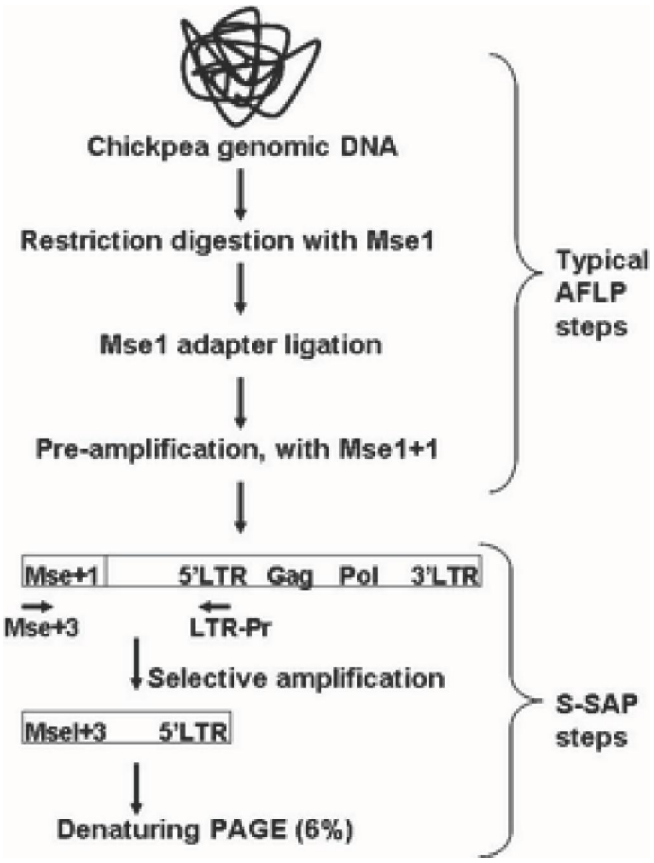

Figure 1 : Schematic representation of the S-SAP technique

Polymorphism is detected as presence/absence of the band, thus S-SAP is usually interpreted as dominant marker system. S-SAP assays have been accomplished in several plant species such as barley[14,25,34], pea[5,19], Wheat[10], oat[35], alfalfa[20], maize[9], sweet potato[29], common bean[8], grapevine[13], tomato and pepper[30], cashew and lettuce[27,28], apple[31] and lotus[15].

Chickpea (*Cicer arietinum* L.) is one of the most important food legumes in the world and Indian subcontinent contributes about 80% to the South-East Asian chickpea production. Legumes such as chickpea have a narrow genetic base therefore, the genetic diversity should be accessed before crop improvement through breeding. In chickpea, several DNA marker techniques have been employed to detect the extent of genetic variability but the high degree of polymorphism could not be revealed. However, the extremely efficient technique S-SAP has not been employed in chickpea due to lack of LTR sequence information. Since, S-SAP has proved to be much more efficient in detecting polymorphism than AFLP in other crops, it was decided to ascertain the effectiveness of S-SAP in detecting polymorphism in chickpea. The LTR-sequence specific primer sequence for S-SAP was taken from *CARE*1 retrotransposon, which was isolated by us earlier[22]

EXPERIMENTAL

Plant material and genomic DNA isolation

Seeds of various Chickpea (*Cicer arietinum* L.) accessions grown in various countries were procured from ICRISAT (International Crop Research Institute for Semi Arid Tropics), Patancheru, Andhra Pradesh, India (TABLE 1). These seeds were grown and the genomic DNA was extracted from young leaves with standard procedures of[1]. The DNA solution was treated with RNase A (Sigma, 100 µg/mL) at 37^{0}C for 20 minutes. The concentration of DNA samples was determined by: Absorption at 260nm × 50 (µg/mL) × dilution factor. The integrity of DNA in the samples was confirmed by 0.7 % (w/v) TAE agarose gel electrophoresis[24].

S-SAP development

Since S-SAP is a modified form of AFLP and many steps are common therefore, AFLP analysis System-I Kit (Invitrogen) was used and AFLP steps were performed according to the protocol of[32]. The S-SAP analysis was carried out as described by[34], with modifications. For this analysis, 150 ng of genomic DNA was digested with 2.5 U *Mse*I for 8 hr at 37°C. The digested sample was incubated at 70°C for 15 min to inactivate *Mse*I. To the digested product *Mse*I adapters were ligated with 1 unit of T4 DNA ligase at 16°C for 4 hrs. The adapter-ligated DNA was diluted five times in 0.1 M TE and 2.5 µl of it was pre-amplified in a PCR reaction containing 10 pmol of pre-amplification primer mix (complementary to adaptor sequences, with one selective nucleotide, namely MseI+N) following procedures of[32]. S-SAP amplicons were generated in 8 µL PCRs, containing 2 µL 25x diluted pre-amplified reaction, 10 pmol of γ^{32}P-ATP (5000 Ci/mmole) labelled LTR primer (5' CTGTGTGGGGGATAGTGTGTTTGT 3'), 10 pmol

TABLE 1 : Cicer arietinum L. accessions used in this study

S. no.	Accession number	Source country
1	ICC2204	SRILANKA
2	ICC2210	ALGERIA
3	ICC10302	COLOMBIA
4	ICC5816	INDIA
5	ICC12237	INDIA
6	ICC1026	IRAQ
7	ICC1164	NIGERIA
8	ICC16530	PAKISTAN
9	ICC12332	TURKEY
10	ICC16833	UGANDA
11	ICC14933	YUGOSLAVIA
12	ICC6263	RUSSIA
13	ICC15802	SYRIA
14	ICC16487	PAKISTAN
15	ICC15518	ALGERIA
16	ICC7272	INDIA
17	ICC12947	INDIA
18	ICC16796	PORTUGAL
19	ICC7571	ISRAEL
20	ICC1422	INDIA
21	ICC4841	MOROCCO
22	ICC3485	JORDAN
23	ICC1422	INDIA
24	ICC7571	ISRAEL
25	ICC8740	AFGANISTAN
26	ICCV2	MYANMAR
27	ICCJG62	INDIA
28	P2	PAKISTAN
29	FLIP82	PAKISTAN
30	BGD72	INDIA

of selective AFLP primer (MseI+3 containing 0.2 mmol dNTPs) and 0.5 units of *Taq* DNA polymerase. Thermal cycling profile was: 10 cycles of 94°C for 1 min, 65°C (reducing by 1°C for each successive cycle) for 1 min and 72°C for 90 sec. This was followed by 23 cycles of 94°C for 30 sec, 56°C for 30 sec and 72°C for 1 min. Three primer combinations LTR—MseI+CTA, LTR—MseI+CAT and LTR—MseI+CTG were used to generate S-SAP fragments. These S-SAP fragments were resolved on 6% denaturing polyacrylamide gel as described by[24].

Analysis of S-SAP data for genetic diversity

S-SAP bands detected by all primer combinations were scored manually. The bands were assigned to the categories depending upon their presence and absence in different accessions. It was assumed that each S-SAP fragment represented a single locus, and polymorphic bands were scored as present (1) or absent (0). The percentage polymorphism was calculated by dividing the number of polymorphic bands by total number of bands scored for an accession.

The data was analyzed with NTSYS-pc software (Numerical Taxonomy and Multivariate Analysis System, Version 1.8) for cluster analysis[23]. The genetic distances were calculated according to the[16] for all possible pairwise comparisons between accessions. Jaccard's similarity index was tried to construct UPGMA-based dendrogram.

RESULTS

The S-SAP technique utilizes LTR sequences, which are highly conserved among the members of a family i.e. for a given family of retrotransposon; LTRs of its individuals are identical to each other. Thus, the success of S-SAP is very much dependent on LTR sequences. In this study, LTR-specific primer sequence was taken from 5'LTR of *gypsy*-like retrotransposon *CARE*1[22]. A total of 30 chickpea accessions from various eco-geographical regions of world were randomly chosen (TABLE 1). The schematic representation of the S-SAP technique is shown in Figure 1. Three different MseI+3 primers (MseI+CTA, MseI+CAT and MseI+CTG) one by one were used in combination with γ^{32}P labelled LTR primer to generate S-SAP amplicons from a preamplified library.

S-SAP data interpretation

The various bands detected by this assay were classified as monomorphic, polymorphic and accession specific (TABLE 2a and 2b). If a band is present in only one accession it is called accession specific. If a band is present or absent in at least two but not all of the accessions it is regarded as polymorphic band. The monomorphic bands are the ones that are present across all the accessions.

Primer combination: MseI-CTA and LTR

Figure 2 shows an S-SAP autoradiograph generated by this primer combination. A total of 83 frag-

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TABLE 2a : Statistical representation of S-SAP data, when all 30 accessions are taken into account.

Primer combinations	Total Bands	Accession specific bands	Monomorphic bands	Polymorphic banda	% Polymorphism	Total accessions
LTR-M-CTA	83	2	78	5	6.02	30
LTR-M-CAT	61	6	56	5	8.19	30
LTR-M-CTG	71	0	69	2	2.81	30

TABLE 2b : Statistical representation of S-SAP data, when the accessions ICC12332, ICC7571, ICC8740, ICCV2, ICCJG62, P2, FLIP82 and BGD72 were excluded.

Primer combinations	Total Bands	Accession specific bands	Monomorphic bands	Polymorphic bands	% Polymorphism	Total accessions
LTR-M-CTA	78	0	78	0	0	22
LTR-M-CAT	56	0	56	0	0	22
LTR-M-CTG	69	5	69	0	0	22

ments were amplified from the pre-amplified library (TABLE 2a and 2b). When all 30 accessions were taken together, the polymorphism was 6%. There were only two accession specific bands, which were present in the accession number ICC8740. When accession numbers ICC12332, ICC7571, ICC8740, V2, JG62, P2, FLIP82 and BGD72 were excluded, the polymorphism dropped to 0% i.e. all accessions except these accessions are monomorphic. The accession ICC8740 was very different from others in its banding pattern.

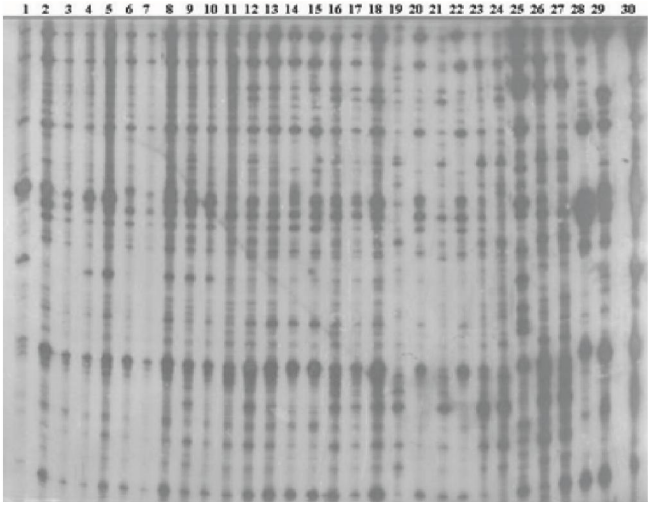

Figure 2 : S-SAP profiles produced by primer combination MseI-CTA and LTR

Primer combination: MseI -CAT and LTR

An overall 8.19% polymorphism was revealed by this combination of primers (TABLE 2a and 2b). Only six accession specific bands were detected, five in accession ICC1422 and one in ICC8740. All accessions except ICC12332, ICC7571, ICC8740, V2, JG62, P2, FLIP82 and BGD72 are monomorphic. i.e. other 22 accessions showed identical banding pattern.

Primer combination: MseI -CTG and LTR

This primer combination generated a total of 71 amplified fragments (TABLE 2a and 2b). The overall polymorphism was 2.81%. When accessions ICC12332, ICC8740, JG62, P2, and BGD72 were excluded, the apparent polymorphism disappeared.

Since twenty two accessions were found to be monomorphic and accessions FLIP82 and BGD72 have identical banding pattern, so a dendrogram could not be generated.

DISCUSSION

One of the major constraints in chickpea crop improvement programs is the limited knowledge of the variability present in its germplasm. Therefore, as a prerequisite for crop improvement programs, there is an urgent need to assess the level of genetic variability. The biodiversity assessment and phylogenetic studies are also essential for preserving both land races and their wild relatives, which are often disappearing rapidly[12].

The techniques such as AFLP are not able to detect much polymorphism in legumes therefore, one has to rely on other techniques such as retrotransposon-based markers. Molecular markers based on retrotransposons have proved to be more informative than non-transposon-based marker methods in many cases tested to date[5,34](Kalendar et al. 1999;). The newly developed retrotransposon-based marker

technology S-SAP is based on fundamentally different biological process to the commonly used marker technologies, but the basic principle of S-SAP is same as of AFLP. In almost all cases LTR sequences of Ty1-*copia*-like retrotransposons are being employed to detect polymorphism in various plants through S-SAP technique[5,10,14,20,21]. Here, LTR sequence of a *gypsy*-like retrotransposon *CARE*1 is being used for S-SAP studies in chickpea. Three combination of primers MseI-CTA+LTR, MseI-CAT+LTR and MseI-CTG+LTR were used to amplify the insertion loci of retrotransposon *CARE*1 in 30 accessions of chickpea, but none of MseI enzyme/primer system revealed more than 8% polymorphism. The percent polymorphism shown by primer combination MseI-CTG+LTR is negligible. The retrotransposon based marker S-SAP used here have proven to be less convincing like AFLP in resolving phylogeny in chickpea[17]. Similar results were obtained by[21] in wheat. But in barley 25-30% and in sweet potato 19-20% increase in the rate of polymorphism was observed with retrotransposon-based S-SAP, in comparison to AFLP analysis. In comparison to AFLP, S-SAP generally yields fewer fragments but higher levels of polymorphism[3,14,34,35]. In *Avena*, the S-SAP technique in combination with AFLP and RAPD generated a saturated map[35]. Plant Ty1-*copia* retrotransposons show a considerable degree of sequence heterogeneity and insertional polymorphism both within and between species but, there is no such report on *gypsy*-like retrotransposons and this could be the reason for detection of low polymorphism. Also, the activity of a given LTR-retrotransposon largely affects the detection of polymorphism by S-SAP. It could be assumed that the insertion of *CARE*1 had taken place in chickpea before the diversification of these chickpea accessions from a common ancestor. Thus the low polymorphism shown in chickpea may be due to antiquity and inactiveness of *CARE*1 retrotransposon[22].

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