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Original Research Article

Molecular Characterization of the Recombinant Inbred Line Population Derived from a *soja* and *max* Soybean Cross

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A B S T R A C T

Soybean is the most important oilseed crop in the world due to its high oil (20%) and protein (40%) content. Productivity of soybean in India is low. Poor genetic variability in soybean is an important factor of low yield. Wide hybridization was attempted between cultivated genotype DS9712 and wild type accession DC2008-1. Isolating from the segregating population in $F_{2:6}$ generation, 206 RILs were developed and tested for two consecutive years i.e. *kharif* 2014 and 2015. Enormous variability was observed among the RILs for phenotypic traits including yield. Genetic polymorphism was studied with 317 SSR markers, out of which 206 were polymorphic (~65% polymorphism). Distribution of the polymorphic markers was not uniform on the chromosomes. Tri-nucleotide repeat motif SSR markers were more polymorphic than others. About 8 markers showed distorted segregation. Linkage map was constructed with 194 markers. Total length of the map was 1823.71cM with an average marker spacing of 9.21cM. The RILs and the molecular map produced in the study will be suitable for mapping and analyzing QTLs for yield and other related traits in soybean.

Keywords

Glycine max, QTL, Recombinant inbred line, Soybean, *soja*, SSR markers

Introduction

Soybean [*Glycine max* (L.) Merr. (2n = 40)] is the most important oilseed crop of the world. It contains high quality protein (~40%), oil (~20%), and a number of neutraceutical compounds such as isoflavons, tocopherol, lecithin, etc. It is used to produce food, feed and a variety of pharmaceutical, cosmetic and industrial items. Cultivation of soybean also enriches the soil by fixing atmospheric Nitrogen. However, yield of soybean in India is lower than the global average (2.5t/ha). Lower level of genetic diversity among Indian soybean germplasm, devoid of gene ic resources in gene pool-2 (GP-2), attack by various biotic and abiotic stresses, use of limited germplasm lines in breeding programs, little or no use of wild type germplasm, etc. are some of the factors for lower yield in India (Yashpal *et al.*, 2015). The wild type soybean G soja harbors several traits which are important for biotic and abiotic stress tolerances. Successful introgression of QTL for useful traits from *G. soja* into *G. max* has already been reported (Concibido, *et. al.*, 2003). Therefore, for introgression of useful QTL from wild species, crosses were made between *Glycine max* and *soja* and advanced segregating generation has been developed.

The generation was advanced through single seed descent (SSD) approach. A set of recombinant inbred lines (RILs) have been isolated in $F_{2:6}$ generation. The specific objective of this study was to characterize the RILs using phenotypic and molecular markers.

Materials and Methods

A cultivated soybean (*Glycine max* L. Merr.) variety DS9712 was crossed with a wild type (*G. soja* Sieb. & Zucc.) accession DC2008-1. Both the parental genotypes were contrasting for all the traits under study. Plants of the cultivated species are erect with sturdy stem and determinate growth, whereas the *G. soja* plants are climbers with indeterminate growth. *G. soja* flowers are very small and purple in color, and produce black seeds. The pods of *G. soja* shatter easily upon maturity. Salient features of the parental genotypes of the RILs are presented below:

About 206 RILs lines were evaluated in Augmented Block Design-II (ABD-II) (Federer, 1956, 1961) along with parents and six standard check varieties (DS9712, Bragg, JS9214, PK1169, EC-439619 and PS1092) in four blocks under field condition at ICAR-IARI, New Delhi. Recommended agronomic practices were followed to raise a good crop. Data were recorded for yield and yield attributing traits such as days-to flowering, days to 50% flowering, days to maturity, plant height, number of primary branches per plant and number of pods per plant. Five single plants in the middle of the row were selected to avoid the border effect to record the data.

For surveying the polymorphism between the parents, SSR markers were picked up from the entire soybean genome. Thus, a set of 317 SSR markers were used for surveying the parental polymorphism. The markers were selected from the consensus map of soybean and sequences were downloaded from the soybase web site (www.soybase.org). The markers were synthesized through local vendors (Sigma-aldrich.com).

Genomic DNA was extracted from young soybean leaves using CTAB procedure (Murray *et al.*, 1980). The healthy leaves from 2-3 weeks old seedlings were collected and wrapped in aluminum foils. Young leaves of each sample (~2 gm) were ground into a fine sample using extraction buffer. The grinded sample was then transferred to micro centrifuge tube containing extraction buffer and, incubated for 1 hr in water-bath at 65^0C with occasional stirring. An equal volume of chloroform: isoamyl alcohol (24:1) was added and mixed thoroughly for half an hour by gentle inversion.

The contents were centrifuged at 12,000 revolutions per minute (rpm) for 20 minutes. The top aqueous solution was carefully transferred to new micro centrifuge tube. Added 5 ml of RNAse to each tube and kept for incubation at 37^0C for half an hour. The contents were centrifuged at 10,000 rpm for 10 minutes. Top aqueous solution was removed and transferred to new micro-centrifuge tube. Approximately 400 µl volume of ice cold Ethanol (100%) was added to it and mixed well and, kept in -20^0C for overnight. The precipitated DNA was centrifuged at 10,000 rpm for 10 minutes to form DNA pellet which was washed with Ethanol (70%). Removed the supernatant and the DNA pellet was air dried at room temperature and the pellet was dissolved in sterile double distilled water.

The PCR was carried out in the 96-well PCR plates with thermal seal and run in a thermocycler (*Applied Biosystem*). The PCR

profile was optimized to have full amplification of the target segment of DNA. The profile of the PCR is given below:

The PCR products were mixed with loading dye before loading into MetaphoreTM gel for size separation.

For size separation through gel electrophoresis, 3 µl of 1x loading dye was added to each PCR tube. A 3 % metaphor gel in 1x TAE buffer with 12 µl ethidium bromide (10mg/ml) for 300 ml of volume was prepared. It was allowed to solidify for about 45-60 minutes. The amplified PCR product was loaded in the prepared gel along with 100 bp ladder purchased from local vendor (Merck and BR Biochem).

The electrophoresis was carried out at 80 V for 2.5-3.00 hours, till the bromophenol blue dye travelled more than 2/3rd the length of the gel. The resolved amplification products were visualized under UV-Transilluminator. The gel was photographed using a Gel Documentation System (AlphaImager-Protien Simple), and subsequently used for scoring.

The data collected from the RILs were analyzed with SPAR software available on web-site (http://www.iasri.res.in) of the ICAR-Indian Agricultural Statistics Research Institute (IASRI), New Delhi.

Results and Discussion

Phenotypic characterization of RIL population

A total of 206 RILs along with the two parental genotypes [DC-2008-1 (*soja*), DS-9712 (*max*)] *a*nd six checks (Bragg, JS9214, PK1169, EC-439619, Pusa9712 and PS-1092) were evaluated in the Experimental Field of ICAR-Indian Agricultural Research Institute (IARI), New Delhi during two consecutive years (*kharif* 2014 and *kharif* 2015). The experiments were laid out in augmented design and genotypes were grown in blocks. High order variability was created among the RILs for all the phenotypic traits (Fig. 1).

Each RIL was grown in 3.0 m long row with a row-to-row spacing of 45cm and plant-to-plant spacing of 10 cm. Standard packages of practices were followed to raise a healthy crop. For collection of phenotypic data, 3 plants were selected from each row avoiding bordering plants, and averaged it.

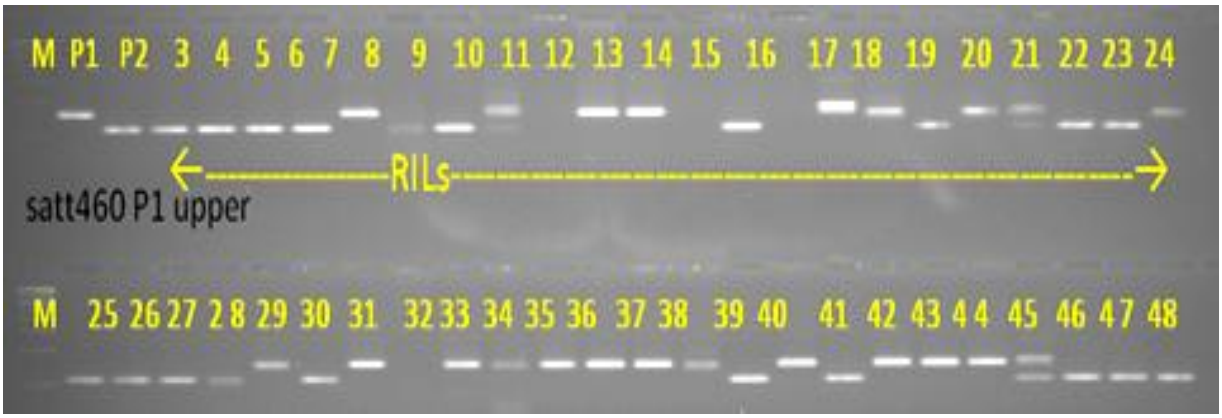
The analysis of variance (ANOVA) of the data was calculated following procedure for augmented design. The ANOVA indicated that there are tremendous variations between the RILs and the parental lines for the traits under consideration (Table 2 and 3) for various yield and other agronomic traits *viz*., flowering time (FT-days required to first flowering in each RIL line from its sowing date), days to 50% flowering (DFF- days required to nearly 50% plants flower in each RIL lines), days to maturity (DM- days required to attend its physiological maturity from its sowing data), plant height (PH- it measured from ground base to the tip of each plant), Number of primary branches per plant (PB), Number of pods per plant (PN), Number of nodes on main stem per plant (NN), Maximum internodal length (MIL), 100-seed weight (SW- it measured by selecting 100 random seeds per plant and weight in g.) and total seed yield per plant (YP).

Morphological observations were recorded on parents and RIL population in both the years of experimentation i.e. *kharif* 2014 and *kharif* 2015 and trait wise performance were analyzed by following standard statistical procedures.

Fig.1 Variability created through inter-specific hybridization

A) RIL wish erect plant habit, B) Variations in leaf size and shape

Fig.2 Patterns of bands in the RILs with SSR marker Satt460



P1: DC2008-1, P2: DS9712. Lane 4-22: Segregating RIL plants. M: Marker ladder

A cultivated soybean (*Glycine max* L. Merr.) variety DS9712 was crossed with a wild type (*G soja* Sieb. & Zucc.)

Genotype	Source	Description
DS9712	Delhi	High yielding popular variety of northern India, erect plant type, determinate and non-twining growth habit, rigid and thick stem, short stem stature, short and broader leaf, low branching, white flower, medium seed size, pod shattering resistant, maturity duration: 110-115 days, and resistant to Yellow Mosaic Virus (YMV) disease.
DC2008-1	USDA	Wild type (*Glycine soja* Sieb. &Zucc.) accession, indeterminate and twining growth habit, weak and thin stem, tall plant stature, small, purple flower, tiny seed, maturity duration: >145 days resistant to YMV disease.

Table.1 List of PCR profile

Step	Activity	Temperature (^{0}C)	Duration (min)	Cycle (No.)
I	Denaturation of template DNA	94	4	1
II	Denaturation	94	1	
III	Annealing	49-55	1	45
IV	Elongation	72	1	
V	Final elongation	72	10	1
	Storing	4	α	

Table.2 Analysis of variance (ANOVA) for yield and other important traits in RILs during *kharif* 2014

Treatment adjusted (degree of freedom: block: 3, treatment: 211, error: 15)										
Source	**DF**	**DFF**	**DM**	**PH**	**PB**	**PN**	**SW**	**YP**	**NN**	**MIL**
TMS	43.87**	54.41**	47.95**	196.23	9.03	646.56	8.49	15.87	13.54	8.31
EMS	3.12	2.02	2.94	19.16	0.67	54.34	0.34	5.19	5.05	0.88
F	23.76	26.98	16.28	10.24	11.38	81.08	24.87	3.06	12.73	13.67
P>F	0.000010							0.009		
Block adjusted (degree of freedom: block:3, treatment: 211)										
BMS	2.19	2.31	9.18	49.10	1.10	21.28	0.48	6.87	11.41	2.08
F	1.36	1.14	3.12	2.57	1.64	0.76	1.41	1.32	1.79	1.46
P>F	0.56	0.36	0.06	0.09	0.22	0.39	0.28	0.30	0.35	0.24

Table.3 Analysis of variance (ANOVA) for yield and other important traits in RILs during *kharif* 2015

Treatment adjusted (degree of freedom: block:3, treatment: 211, error: 15)										
Source	**DF**	**DFF**	**DM**	**PH**	**PB**	**PN**	**SW**	**YP**	**NN**	**MIL**
TMS	96.18**	53.21**	49.52**	264.54	6.21	11208.56	9.34	48.25	11.23	9.09
EMS	5.32	2.20	2.74	29.16	1.63	204.34	4.28	5.28	4.47	0.77
F	21.12	25.68	19.81	9.61	8.18	55.458	9.29	6.06	13.53	14.19
P>F	0.000013							0.089		
Block adjusted (degree of freedom: block: 3, treatment: 211)										
BMS	4.49	3.61	5.08	42.34	1.16	102.28	1.65	7.201	11.41	2.61
F	1.16	1.74	1.78	1.33	1.45	0.79	1.41	1.35	1.79	1.24
P>F	0.55	0.26	0.16	0.24	0.26	0.54	0.28	0.33	0.35	0.46

In each RIL along with parental lines, three individual random plants were selected avoiding border effect plants from each replication and averaged it. The averages values are used to assess its statistical analysis for phenotyping.

Phenotypic evaluation of the RIL population was carried out to ensure stabilization of the population for various traits under study. The study indicated stability of the population pointing its fitness for analysis and mapping of the QTL.

Parental polymorphism studies

For surveying the level of genetic polymorphism between the two parents i.e. DC2008-1 and DS9712, a set of 317 simple sequence repeat (SSR) markers were selected at random from across the soybean genome with an average about 16 markers per chromosome (i.e. 8 markers per arm of each 20 chromosome). Among the 317 SSR markers, 206 were found to be polymorphic between the parents i.e. the level of genetic polymorphism between the two parents were 64.98%. Such higher level of polymorphism was observed due to existence of genetic diversity between the parental genotypes originating from two different species contrasting phenotypically.

However, distribution of the polymorphic markers was not uniform across the chromosome; some chromosome had found more polymorphic markers than others. Chromosome Nos. 10 and 20 had the highest level of polymorphism (90.91%) while the chromosome number 1 and 11 had the least (50.00%).

Power of the SSR markers in detecting the polymorphism level found to vary with the type of motif and the number of repeats in a particular motif. The tri-nucleotide motifs were more polymorphic (54.06%) than dinucleotide (37.79%) or polynucleotide motifs (8.13%).

Further it was observed that the level of polymorphism detected between the two species also varied with the number of times a particular motif is repeated. Out of 111 mono-morphic SSR markers, 66 had repeat motif with less than 20 repeat units. On the other hand, among 206 polymorphic markers, 111 had motifs with 20-30 or more repeats. Thus, it appeared that the SSRs with 20-30 repeats, i.e. say ATT20, ATT25, ATT30, etc are relatively more polymorphic than others.

Marker segregation analysis

Molecular genotyping of the RILs was done with 194 SSR markers. Segregation data of each marker was subjected to χ^2 (Chi-square) test for goodness of fit to 1:1 ratio. Out of 206 polymorphic markers used, all 194 markers showed a goodness of fit to the expected segregation ratio of 1:1 at significance level of $P< 0.05$, while 4 markers showed distorted segregation and high missing datapoint, which were eliminated from further analysis. Pattern of bands obtained in the RILs is shown in a representative gel (Fig. 2).

A framework linkage map of the 20 chromosome (n = x = 20) was developed using the 194 SSR markers that covered a total map length of 1823.71 cM. The markers covered the entire soybean genome near evenly. Average distance between adjacent markers was 9.21cM, which is good enough for QTL mapping and analysis. Number of marker on a chromosome ranged from 6 to 13. Lowest number of marker i.e. 6 was observed on chromosome No. 13 and 15 and highest number i.e. 13 was observed on chromosome No. 2 and 12. The map length of chromosome Nos. 2 and 12 was 105.03cM and 105.15 cM, respectively. Highest map length of the chromosome was observed on chromosome Nos. 8 (147.04cM) followed by chromosome Nos. 10 (124.36cM) and 13 (117.40cM) with average distance between adjacent marker was 13.37, 12.44 and 19.57cM respectively, whereas lowest map length was noticed on chromosome Nos. 15 (36.31cM) followed by chromosome Nos. 1 (44.61cM) and 5 (72.20cM) with average distance between adjacent marker was 6.05, 4.96 and 6.56cM respectively.

The map constructed in this study is comparable with other similar maps published elsewhere. It contains the markers nearly uniformly across the genome. The RILs produced in this study will be utilized for mapping and analyzing QTL for other important traits in soybean.

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