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Research Article

Genetic mapping and validation of SSR markers linked to leaflet shape in soybean using a recombinant inbred line population

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Abstract
The leaflet shape in soybean is an important trait and is genetically controlled by a single *Ln* locus. The narrow leaflet shape is associated with high photosynthetic efficiency and a higher number of seeds per pod. The current study involves the genetic mapping of SSR markers linked to leaflet shape in a $F_{2:8}$ soybean recombinant inbred population and validation in different genetic backgrounds. A total of 240 SSR markers were used for bulked segregant analysis and six SSR markers (Sat_105, BARCSOYSSR_20_0813, BARCSOYSSR_20_0839, BARCSOYSSR_20_0846, BARCSOYSSR_20_0850, and BARCSOYSSR_20_0855) located on chromosome 20 were found to be linked to leaflet shape locus. The linkage analysis distributed these six SSR markers and leaflet shape (*Ln*) locus in one linkage group within a map distance of 11.5 cM. BARCSOYSSR_20_0839 and BARCSOYSSR_20_0846 were the two most tightly linked SSR markers and flanked the *Ln* locus at a distance of 0.3 cM and 1.8 cM, respectively. The markers selection efficiency varied from 86.6 to 99.4 per cent. The specificity of tightly linked SSR markers for leaflet shape was confirmed by validation on 24 different soybean genotypes. The SSR markers successfully distinguished the narrow leaflet genotypes from broad leaflet genotypes, indicating their specificity to leaflet shape locus and thus offer scope for further exploitation.

Key words: Soybean, leaflet shape, SSR markers, *Ln*, genetic mapping, validation

INTRODUCTION

Soybean is an important leguminous seed crop and is a rich source of both edible oil and protein. Soybean seed contains approximately 40% protein and 20% oil and is therefore considered a dual-use crop. In recent years, demand for soybean is increasing steadily due to its food, feed and industrial uses which eventually led to concerted efforts to develop high yielding cultivars. Yield in soybean is influenced by many morphological characters *viz.,* plant height, days to maturity, the number of branches, the number of pods per plant, the number of seeds per pod, seed weight etc. (Yadav *et al.,* 2009; Li *et al.,* 2020). The canopy structure and population density are two important factors associated with leaflet shape that is known to affect the yield (Bianchi *et al.*, 2020). Soybean has two types of leaflet shapes *viz.,* broad (ovate) and narrow (lanceolate). Soybean genotypes with narrow leaflet shapes have better light distribution through the canopy and higher photosynthetic rates compared to genotypes with broad leaflet shapes (Wells *et al.*, 1993; Suh *et al.*, 2000). You *et al.* (1995) reported that narrow leaflet genotypes produce more yield at higher population densities and broadleaf genotypes at low population densities.

The leaflet shape in soybean is under the control of a single gene (*Ln*). The dominant (*Ln/Ln*) locus governs broad (ovate) leaflet shape while recessive locus (*ln/ln*) governs narrow (lanceolate) leaflet shape (Bernard and Weiss, 1973; Sawada, 1988). The allele for narrow leaflet shape exhibits a positive pleiotropic effect on the

number of seeds per pod and results in the increased number of four-seeded pods (Weiss, 1970; Jeong *et al.*, 2012). The pleiotropic effect of *ln* locus helps to select the plants with more seeds per pod indirectly. However, leaflet shape in soybean may vary from the bottom to the top of the plant and is also influenced by environmental conditions, making the selection difficult (Sawada, 1992). The use of tightly linked molecular markers would allow plant breeders to practise indirect selection for the target gene in a segregating population with increased reliability (Collard *et al.*, 2005). Kim *et al.* (2005) identified many QTLs associated with leaflet shape (length to width ratio) located on different soybean chromosomes in two RIL populations. However, the individual contribution of these QTLs to leaflet shape phenotype was small and only one QTL located on LG A2 (Chr. 8) was considered as putative QTLs for leaflet ratio in multiple linkage group regression (MLGR) analysis (Kim *et al.*, 2005). In different studies, leaflet shape locus was mapped on different chromosomes. Jeong *et al.* (2011) mapped the narrow leaflet locus (*ln*) using SSR markers on chromosome 20. In another study, Wang *et al.* (2019) mapped 11 QTLs for leaf shape on four chromosomes (Chr. 3, 12, 14 and 19) with phenotypic variation ranging from 2.71 to 13.77 per cent. Thus, there is a need to validate these markers in the genotypes/populations before utilization in marker assisted selection (MAS). Bulked segregant analysis (BSA) is a gene mapping strategy that has been successfully used to rapidly identify the molecular markers linked to the target genes (Rani *et al.,* 2017; Kang and Milan, 2010). Therefore, the aim of this study was to identify and map the SSR markers linked to leaflet shape in a RIL population and validation of tightly linked markers in different soybean genetic backgrounds for use in MAS.

MATERIALS AND METHODS

For mapping, 180 $F_{2:8}$ recombinant inbred lines (RILs) were used. The RIL population was developed by hybridizing a soybean variety (DSb-12) having narrow (lanceolate) leaflets with soybean genotype (PK564) having broad (ovate) leaflets. The F_1 plants were selfed to produce F_2 seeds and thereon single seed descent method was followed upto eight generation to develop the RIL population. To check the specificity of molecular markers for leaflet shape in different genetic backgrounds, a total of 24 soybean genotypes were used, which included 10 genotypes having narrow leaflet shape and 14 genotypes with broad leaflet shape. The RILs, two parents and other soybean genotypes were grown under natural filed conditions and were evaluated for leaflet shape on the basis of the leaflet shape index ratio (leaflet length to width ratio) as described by Chen and Nelson (2004). The maximum length and width of the leaflets in the trifoliate leaf from the 4th node was taken for ratio measurement. The average value of three leaflets was used for classification into broad or narrow types. The leaflets with a ratio of 2.5 and more were considered as narrow and with less than 2.5 were considered as broad type.

Leaf tissues were collected from all plants individually and were ground in liquid nitrogen. DNA was isolated from 100 mg of powdered leaf tissue using the DNeasy Plant Mini kit (Qiagen, USA). The quality and quantity of DNA samples were estimated on Nanodrop ND 1000 spectrophotometer (Thermo Scientific, USA). DNA samples were diluted to 10ng/µl for PCR analysis. Bulked segregant analysis (BSA) method described by Michelmore *et al.* (1991) was employed to identify the SSR markers linked to leaflet shape phenotype. The two contrasting DNA bulks for leaflet shape were made from randomly chosen RILs. An equal quantity of DNA from 10 RILs having narrow leaflet shape were mixed to form narrow leaflet DNA bulk and an equal quantity of DNA from 10 RILs having broad leaflet shape were mixed to form broad leaflet DNA bulk. The two contrasting DNA bulks along with DNA from both the parents were screened with 240 SSR primers (Song *et al.*, 2004; Song *et al.*, 2010). For screening, 9-15 SSR primers were randomly chosen from each soybean chromosome. The identified polymorphic SSR primers for two parents and bulks were considered as putative markers linked to leaflet shape and were screened on the whole RIL mapping population for linkage analysis.

The PCR amplification was performed on a thermal cycler (Eppendorf, Germany) and 20 *µ*l PCR reaction mixture included 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.0 mM $MgCl_2$, 100 *µ*M of each dNTP, 0.75 U *Taq* DNA Polymerase (Jonaki Taq), 0.2 *µ*M of each SSR primer pair and 40 ng of genomic DNA. The PCR were performed using the following thermal profiling conditions: 95^{o}C for 4 min (1 cycle); 94^{o}C for 30 sec, 56-58^{o}C for 30 sec, 72^{o}C for 30 sec (45 cycles); 72^{o}C for 10 min (1 cycle). The PCR products were resolved on 2% agarose (w/v) gels in 1X TBE buffer at 80V (5 V cm^{-1}) for 2 h. Gels were stained with ethidium bromide and photographed on a digital gel documentation system (Syngene, USA).The SSR genotyping data and leaflet shape phenotyping data of 180 RILs was used to establish the linkage between leaflet shape and SSR markers by using the JoinMap program, version 3.0 (Van Ooijen, 2006). A logarithm of odds (LOD) score of >3 was used to identify the linkage and Haldane's mapping function was used to convert the recombination frequencies into map distances in centiMorgan (cM).

RESULTS AND DISCUSSION

The parent DSb-12 had a leaflet shape index ratio of 2.8 and was classified as a narrow (lanceolate) type, while it was 1.9 for PK564 and therefore classified as broad (ovate) type (**Fig. 1**). Earlier, Sawada (1992) reported that a leaflet shape index ratio of 2.5 can be used to differentiate between broad and narrow leaflet shapes. The $F_{2:8}$ RIL population developed from these parents showed segregation for leaflet shape. Based on leaflet shape index ratio, 93 RILs were classified as broad and 87 as narrow type, fitting a 1:1 ratio ($\chi^2 = 0.20$, $P = 0.654$) for a monogenic trait, as expected. The monogenic inheritance of leaflet shape in soybean has been previously reported (Bernard and Weiss, 1973; Sawada, 1988). For identifying

the molecular markers linked to leaflet shape, a total of 240 SSR markers randomly chosen from different chromosomes were used for BSA. Initially in BSA, only two SSR markers Sat_105 and BARCSOYSSR_20_0855 located on chromosome 20 were found polymorphic. Additional SSR markers located between these two SSR markers (Sat_105 and BARCSOYSSR_20_0855) were selected from Song *et al.* (2010) and screened to get more polymorphic markers. Finally, six SSR markers (Sat_105, BARCSOYSSR_20_0846, BARCSOYSSR_20_0850, BARCSOYSSR_20_0813, BARCSOYSSR_20_0839 and BARCSOYSSR_20_0855) showed polymorphism. All SSR markers exhibited a codominant banding pattern and amplified alternate alleles from both the parents (**Table 1**). The amplification pattern of three SSR markers is shown in **Fig. 2**. All six polymorphic SSR markers were further screened in the 180 $F_{2:8}$ RILs for linkage analysis and all SSR markers were found linked with leaflet shape locus. A few recombinant genotypes were also observed between the SSR markers and leaflet shape locus (**Table 2**). The number of recombinants varied from a minimum of one (BARCSOYSSR_20_0839) to a maximum of 24 (Sat_105). The linkage analysis distributed all the six SSR markers and leaflet shape locus (*Ln*) in one linkage group within a map distance of 11.5 cM (**Fig. 3**). The SSR markers BARCSOYSSR_20_0855,

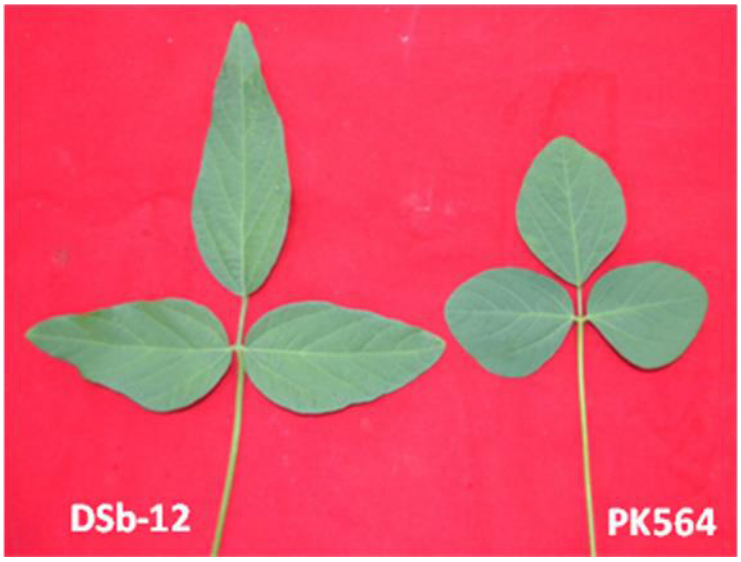

Fig. 1.The leaflet shape of the parent DSb-12 (narrow leaflet) and PK564 (broad leaflet) used for developing the RIL mapping population in soybean

Table 1.The details of new SSR markers mapped to leaflet shape (Ln) locus in soybean

SSR marker	Primer sequence (5'-----3')	Approximate alleles size (bp)
BARCSOYSSR_20_0855	F: GCGTGCAACATATGACACCATAAAT R: GCGTGAGGAGGTTCAAAAATAACAT	220 &250
BARCSOYSSR_20_0850	F: AAAGTATCAATACCCGGCCC R: TTAGCAATGGTTTTGGGGAG	110 & 98
BARCSOYSSR_20_0846	F: GGCCAAATCTTCTTTTGGGT R: AAGCAAGGTCGGTGTTTTTG	140&102
BARCSOYSSR_20_0839	F: AAACACACTCGATGGCTTGA R: ATCCGTTGCTCACCCAATTA	292& 278
BARCSOYSSR_20_0813	F: TGAATAACAAGGGGCGAAAG R: ATGGAAACGCAGTTTGAAGC	255 & 240
Sat_105	F: TTCCATACAAGATATCAAGTGAATTG R: GCTCCCCTACATTGGTAGTAAA	300&260

F: Forward primer; R: Reverse Primer

Table 2. Segregation of SSR markers in 180 recombinant inbred lines (RILs) of the soybean cross 'PK564' × 'DSb-12'

SSR marker	Marker alleles segregation in RIL population						Marker selection accuracy	
	Br/1+	Br/2+	Na/1+	Na/2+	χ^{2*}	*P* value	Frequency	%
BARCSOYSSR_20_0855	85	8	2	85	0.80	0.370	170/180	94.4
BARCSOYSSR_20_0850	88	5	2	85	0.20	0.654	173/180	96.1
BARCSOYSSR_20_0846	88	5	1	86	0.35	0.550	174/180	96.6
Ln_at013	90	3	0	87	0.20	0.654	177/180	98.3
BARCSOYSSR_20_0839	92	1	0	87	0.02	0.881	179/180	99.4
Ln_atre04	92	1	1	86	0.00	1.00	178/180	98.8
Ln_at008	92	1	1	86	0.00	1.00	178/180	98.8
BARCSOYSSR_20_0813	86	7	4	83	0.20	0.654	169/180	93.8
Sat_105	77	16	8	79	1.42	0.232	156/180	86.6
BARCSOYSSR_20_0839 + BARCSOYSSR_20_0846	93	0	0	87	0.00	1.00	180/180	100

Br: broad leaflet phenotype; Na: narrow leaflet phenotype; 1+: presence of marker allele 1; 2+: presence of marker allele 2
*Chi-square values based on the expected Mendelian segregation ratio of 1:1

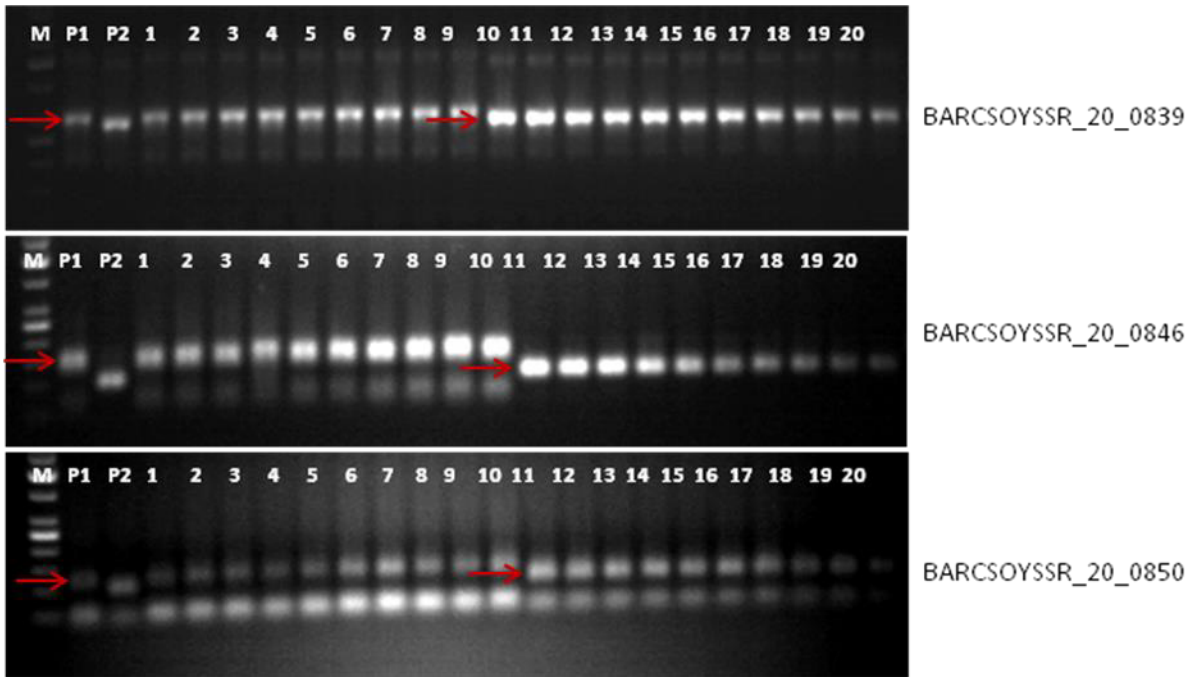

Fig. 2. Amplification pattern of SSR markers

(a) BARCSOYSSR_20_0839 (b) BARCSOYSSR_20_0846 and (c) BARCSOYSSR_20_0850 on parents and 20 RILs of the soybean cross 'PK564' × 'DSb-12'.

Legends: M: 50 bp DNA molecular weight marker; P1: Broad leaflet parent (PK564); P2: Narrow leaflet parent (DSb-12); lane 1-10: RILs having broad leaflets; and lanes 11-20: RILs having narrow leaflet shape

BARCSOYSSR_20_0850, and BARCSOYSSR_20_0846 were located on one side of *Ln* locus and markers BARCSOYSSR_20_0839, BARCSOYSSR_20_0813 and Sat_105, were located on the other side of *Ln* locus. The SSR marker BARCSOYSSR_20_0839 was the most tightly linked marker and was located at a distance of 0.3 cM from *Ln* locus (**Fig. 3**). Another tightly linked SSR marker BARCSOYSSR_20_0846 flanked the *Ln* locus at a distance of 1.8 cM. For successful utilization of markers, the accuracy and specificity of the markers for the target trait are very important. In the current study, markers selection efficiency ranged from a minimum of 86.6 per cent (Sat_105) to a maximum of 99.4 per cent (BARCSOYSSR_20_0839) (**Table 2**). By using the flanking SSR markers BARCSOYSSR_20_0839 and BARCSOYSSR_20_0846, the target phenotype selection efficiency was increased to 100 per cent (**Table 2**). The use of flanking markers greatly increase the reliability of the markers to predict the phenotype because the possibility of error would only result from

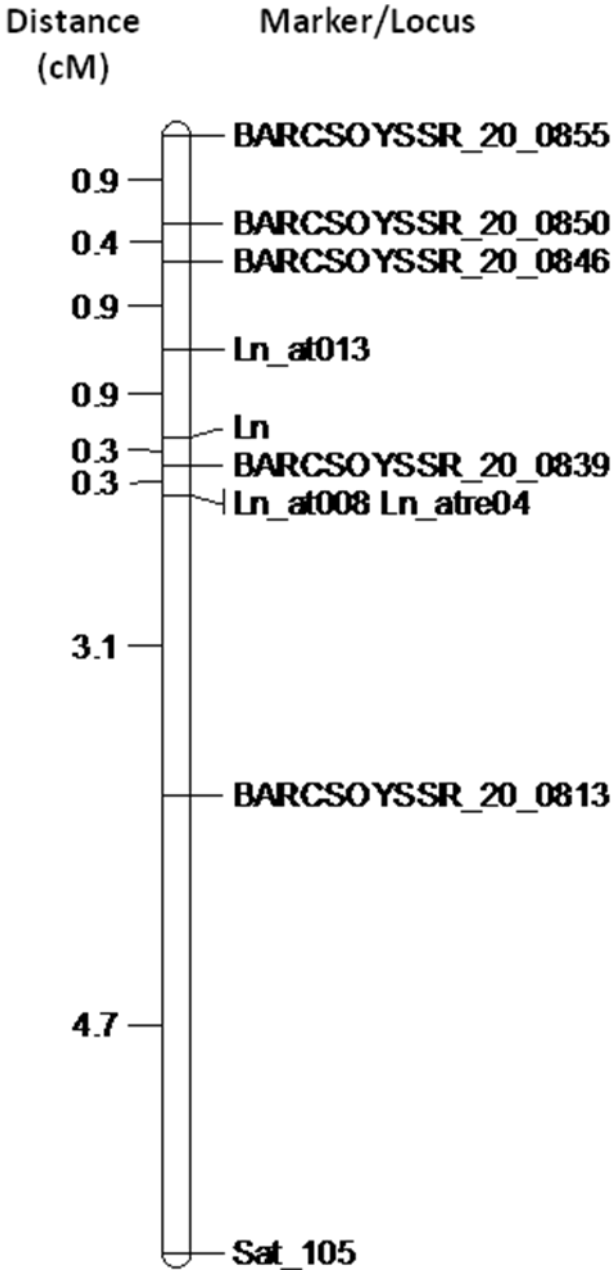

Fig. 3. A genetic linkage map showing the SSR markers linked to leaflet shape locus (*Ln*) on chromosome 20 in soybean. Markers were mapped in 180 RILs of the soybean cross 'PK564' × 'DSb-12'

double recombination and the chance of this occurring in a short segment of the chromosome is very rare (Collard and Mackill, 2008). Many studies have highlighted the use of flanking markers in comparison to single markers in breeding programs increased the selection efficiency (Young and Kelly, 1997; Cherukuri *et al.*, 2005).

Previously, QTLs have been reported for leaflet shape on chromosome 8 (Kim *et al.*, 2005) and on chromosomes 3, 12, 14, 16 and 19 (Wang *et al.*, 2019). In the current study, SSR markers were mapped on chromosome 20 and no SSR marker located in these QTL regions was found polymorphic, indicating that leaflet shape locus is not controlled by these QTLs. Earlier, Jeong *et al.* (2011) had also reported 14 SSR markers linked to *Ln* locus on chromosome 20. The markers earlier reported by Jeong *et al.* (2011) were also validated in the current study. However, only three markers (Ln_at013, Ln_atre04, Ln_at008) were found polymorphic, indicating that markers mapped in one specific population may not be polymorphic in other genetic backgrounds and therefore, markers should always be validated in different genetic backgrounds before employing them for MAS. The failure of markers mapped in one population to select the target phenotype in other genetic backgrounds has been reported in many other studies (Sharp *et al.*, 2001; Prabhu *et al.*, 2004; Bernardo *et al.*, 2013; Gupta *et al.*, 2015). These polymorphic markers (Ln_at013, Ln_atre04, Ln_at008) were further screened in the entire RIL population to establish their linkage with other SSR markers and *Ln* locus (**Table 2**). The markers Ln_atre04 and Ln_at008 were mapped at a distance of 0.6 cM from *Ln* locus and marker Ln_at013 flanked at a distance of 0.9 cM (**Fig. 3**). The SSR marker Ln_atre04 behaved like a dominant marker and amplified only allele linked to broad leaflet shape and null allele was observed for narrow leaflet shape, thereby reducing its utility is MAS.

For MAS, it is essential to check the effectiveness of the molecular markers in different genetic backgrounds. The specificity of four tightly linked SSR markers (BARCSOYSSR_20_0839, Ln_at013, Ln_at008 and

Table 3. Validation of SSR markers tightly linked to leaflet shape on different soybean genotypes

S. No.	Genotype	Pedigree	Leaflet shape Phenotype	BARCSOYSSR _20_0839	Ln_at08	Ln_at013	BARCSOYSSR _20_0846
1	JS93-05	Selection from PS 73-22	Narrow	292 bp	300 bp	320 bp	140 bp
2	PS-1347	PK472 x PS1024	Narrow	292 bp	300 bp	320 bp	140 bp
3	AMS-358	Mutant of JS93-05	Narrow	292 bp	300 bp	320 bp	140 bp
4	JS21-80	-	Narrow	292 bp	300 bp	320 bp	140 bp
5	DSb-12	JS335 x PS73-7	Narrow	292 bp	300 bp	320 bp	140 bp
6	JS95-60	Selection from PS 73-22	Narrow	292 bp	300 bp	320 bp	140 bp
7	DSb-32	-	Narrow	292 bp	300 bp	320 bp	140 bp
8	AMS-475	Mutant of JS93-05	Narrow	292 bp	300 bp	320 bp	140 bp
9	PS-1024	PK308 x PK317	Narrow	292 bp	300 bp	320 bp	140 bp
10	TS20-5	PK564 x JS93-05	Narrow	292 bp	300 bp	320 bp	140 bp
11	PS-1225	PK515 x PK327	Broad	278 bp	280 bp	300 bp	102 bp
12	PK-416	UPSM534 x S38	Broad	278 bp	280 bp	300 bp	102 bp
13	PK-472	Hardee× Punjab-1	Broad	278 bp	280 bp	300 bp	102 bp
14	JS97-52	PK327 x L129	Broad	278 bp	280 bp	300 bp	102 bp
15	SL-744	SL457 x SL459	Broad	278 bp	280 bp	300 bp	102 bp
16	SL-958	SL525 x SL706	Broad	278 bp	280 bp	300 bp	102 bp
17	PS-1092	PK327 x PK416	Broad	278 bp	280 bp	300 bp	102 bp
18	MACS-1520	EC241780 x MACS 330	Broad	278 bp	280 bp	300 bp	102 bp
19	Punjab-1	Selection from Nanking Variety	Broad	278 bp	280 bp	300 bp	102 bp
20	Hardee	D49-772 x Improved Pelican	Broad	278 bp	280 bp	300 bp	102 bp
21	Bhatt	Indigenous native variety	Broad	278 bp	280 bp	300 bp	102 bp
22	Harasoya	Himso-1520 × Bragg	Broad	278 bp	280 bp	300 bp	102 bp
23	Pusa-5	-	Broad	278 bp	280 bp	300 bp	102 bp
24	PS-1042	Bragg x PK 416	Broad	278 bp	280 bp	300 bp	102 bp

BARCSOYSSR_20_0846) was confirmed by amplification on 10 genotypes having narrow leaflet shape and 14 genotypes with broad leaflet shape. All four SSR markers successfully distinguished the narrow leaflet and broad leaflet genotypes (**Table 3**). This indicated that these SSR markers are highly specific to leaflet shape and can be successfully employed for MAS. The use of tightly linked molecular markers to indirectly select agronomically important traits can improve selection efficiency and drastically reduce the time of traditional crop breeding programs (Collard *et al.*, 2005).

Many studies have reported that narrow leaflet shapes can be utilized in soybean breeding to improve the canopy structure and photosynthetic rate of soybean cultivars for genetic gains (Wells *et al.*, 1993; Suh *et al.,* 2000). In addition, narrow leaflet cultivars tend to have an increased number of seeds per pod compared to cultivars with broad leaflets in soybean (Dinkins *et al.*, 2002; Jeong *et al.*, 2012). Despite its effect on yield, narrow leaflet shape has not been significantly utilized in Indian soybean breeding programs. The leaflet shape in soybean is susceptible to environmental conditions and varies with the growth stage of plant (Sawada, 1992). Therefore, tightly linked molecular markers identified in this study would serve as an important genetic resource and would be helpful in the MAS of narrow leaflet phenotype to develop soybean cultivars with improved canopy structure and higher yield.

Eight markers were mapped to leaflet shape (*Ln*) locus in soybean using a RIL population. These SSR markers covered a total genetic distance of 11.5 cM and four markers ((BARCSOYSSR_20_0839, Ln_at013 and Ln_at008 and BARCSOYSSR_20_0846) were located at less than 1 cM from *Ln* locus. These four tightly linked markers were also validated on 24 diverse soybean genotypes. These SSR markers would serve as an important genetic resource for the genetic improvement of soybean.

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