UDC 575.633
https://doi.org/10.2298/GENSR2302631A
Original scientific article

MOLECULAR CHARACTERIZATION OF SEED LONGEVITY IN SOYBEAN [*Glycine max* (L.) Merrill] USING SSR MARKERS

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Adsul A. T., V. P. Chimote, D. S. Thakare, M.P. Deshmukh (2023). *Molecular characterization of seed longevity in soybean* [*Glycine max* (L.) Merrill] *using SSR markers.* - Genetika, Vol 55, No.2, 631-645.

Soybean is the most important oilseed in the tropical and subtropical parts of the world unfortunately has poor competence for seed longevity. It's strongly affected by environment (Temperature and RH) during seed formation, harvest and storage. Tagging of seed longevity associated markers in the crosses (Birsa Soya1 × DS-228) which are good and poor storer genotypes resp. by using 26 SSR primers in genotypes contrasting for these traits in soybean. The polymorphism was observed between genotypes differing for seed longevity trait for six primer pairs, *viz*; Sat_162, Satt632, Sat_202, Satt523, Satt184 and Satt460, among them seed coat hardiness QTL, *SCH 1-2* linked Satt632 amplified distinct markers. Bulked segregant analysis was carried out by using SSR markers in segregating 93 F2 populations. The Satt632-237 bp marker was further validated in individual F2 plant population. The genotype, Birsa Soya1 and it segregants Satt632-237 bp marker was found to be closely associated with high seed longevity in soybean. Hence, the primer Satt632 is impending marker for marker assisted breeding for high seed longevity marker to be linked to seed coat hardiness loci *qSCH1-2* like gene present on chromosome A2. Seed longevity trait is governed by one or few gene with observed segregation ratio of 3.65:1 as against expected segregated ratio 3:1. Candidate

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gene analysis at *qSCH1-2* loci, indicated for role of either a brassinosteriod regulated protein or pentatricopeptide repeat gene to regulate seed longevity.

Keywords: Accelerated aging, Seed Coat Hardiness, Seed longevity, SSR, Soybean

INTRODUCTION

Soybean [*Glycine max* (L.) Merrill] is one of the richest and cheapest sources of protein and staple in the diet of people in numerous parts of the world. Soybean, with over 40 percent protein and 20 percent oil and for its multiple uses and is one of the world's most important economic oilseed crops and is recognized as most important grain legume in the world in terms of total production and international trade. Seed is a basic input for agriculture productivity and germplasm conservation. Seed quality predominantly comprises of germination and vigour which are quintessential for successful stand establishment. On the other hand, seed industries are thriving to improve the crop yield through control of seed vigour (NAIK *et al*., 2019; KUMAR *et al*., 2016). The best seed yields are achieved on fertile soil. Seed longevity is a quantitative trait and is strongly affected by the environment during seed formation, harvest and storage (NAIK *et al*., 2019; CLERKX *et al*., 2004; KOSEV *et al*., 2022). The longevity of seeds in storage is influenced by four major factors *viz*., genetic constitution, seed quality trait during storage, moisture content or ambient relative humidity, and temperature of storage environment. The variation in speed of seed deterioration of soybean varieties is a genetic trait. Genetics provides a powerful approach to identify the physiological and molecular basis of phenotypic traits such as seed longevity and other quality factors. Soybean genotypes differ in their ability to maintain seed longevity (WIEN *et al*., 1981). Research carried out on soybean seed quality during storage and reported that the germination of soybean varieties decreased during storage irrespective of varieties, threshing and processing methods and storage containers. A number of seed characters such as seed size, percent hard seededness, seed coat thickness and permeability, electrical conductivity of the seed leachet, hull percentage, oil content etc., are associated with seed quality in soybean and were shown to be under genetic control (POTTS *et al*., 1978; VERMA *et al*., 1987).

Soybean seed is thus highly susceptible to both pre-harvest and post-harvest damages which adversely affect its longevity. Detrimental effects of allowing soybean seed to remain in the field after reaching physiological maturity have been known for many decades. Weather conditions during the post-maturation pre-harvest period have a great influence on the quality of the harvested seed (DELOUCHE, 1980). The deterioration of seed vigor, as well as viability due to high temperature, high relative humidity and frequent or prolonged rainfall play a critical role affecting soybean seed quality in the tropics (BHATIA *et al*., 1993; KEIGLEY and MULLEN, 1986; MONDRAGON and POTTS, 1974; TEKRONY *et al*., 1980). Post-harvest extended storage of seeds also results in gradual loss of seed viability (WILSON and MCDONALD, 1986). Seed viability is often reduced below the minimum standards prior to planting time and loss of germination potential is acute in tropical and sub-tropical regions as compared to temperate environments (BHATIA *et al*., 1996; KHARE *et al*., 1996).

Seed longevity is the associated with hard seededness; a quantitative trait influenced by several major QTLs. Screening for seed quality is not easily and accurately recordable as its expression is highly sensitive to environmental fluctuations. Therefore molecular approaches can

be used to tackle the problem of longevity for screening the available germplasm. Significant progress in soybean genomics has provided a deeper insight for targeting important genes and quantitative trait loci (QTL) for a number of agronomic traits in soybean have been mapped. High resolution genetic linkage maps provide a tool for genetic dissection and characterization of many quantitatively inherited seed quality traits in soybean (CREGAN *et al.*, 1999; KEIM *et al*., 1990a; KEIM, 1990b; LARK *et al*., 1993; SONG *et al*., 2004). There are a few reports of seed longevity linked molecular markers located on different linkage groups (CHANGRONG *et al*., 2006; CREGAN *et al*., 1999; DARGAHI *et al*., 2014; HOSAMANI *et al*., 2013; SAKAMATO *et al*., 2004; SINGH *et al*., 2008) had reported four SSR markers (Satt538, Satt285, Satt600 and Satt434) significantly associated with seed longevity in soybean.

Molecular gene markers have brought phenomenal changes in the area of plant biotechnology by their ability to produce unique DNA profiles in various crops. Availability of molecular marker technology has made possible the genetic dissection and characterization of many quantitatively inherited seed quality traits in soybean. Restriction fragment length polymorphisms (RFLPs) were utilized to identify several major quantitative trait loci (QTLs) in soybean influencing hard seededness in an interspecific soybean population (KEIM *et al*., 1990). Molecular markers were utilized to identify several genomic regions significantly associated with seed protein, oil, seed weight and sucrose content in different intraspecific soybean population (MANSUR *et al*., 1990; BRUMMER *et al*., 1997) and molecular studies support the complex genetic nature of seed longevity.

Microsatellites or Simple Sequence Repeat (SSR) markers are highly polymorphic, abundant and distributed throughout the genome (CREGAN *et al*., 1999). In one of the study SSR markers are reported to be associated with seed coat permeability and electrolyte leaching and seed longevity in an F2:3 soybean population in a cross involving good and poor storer genotypes (SINGH *et al*., 2008). Marker assisted selection (MAS) using DNA markers instead of phenotypic assays reduces cost and increases the precision and efficiency of subsequent selection steps applied in breeding. In this context, the present study made an attempt to characterize a set of good and poor seed longevity soybean genotypes with SSR markers.

MATERIALS AND METHODS

Plant Materials

The seeds of four genetically diverse genotypes (DS-228, MAUS-71, Birsa Soya1 and Kalitur) obtained from the Soybean Breeder, Agricultural Research Station, Kasbe Digraj, Sangli (MH) India. These genotypes were crossed to produce four combinations *viz., DS-228 × Kalitur* (C-I), *DS-228 × Birsa Soya-1* (C-II), *MAUS-71 × Birsa Soya-1* (C-III) and *MAUS-71 × Kalitur* (C-IV). The segregating F2s mapping populations (93 individual plants) derived from the cross between DS-228 (Low seed longevity) and Birsa Soya1 (High seed longevity) were used for the genotyping and phenotyping in soybean (Delouche and Baskin, 1973). The laboratory work and evaluation of experimental field trial was conducted by using randomized complete block design with three replications at Post Graduate Institute, Botany Research Farm and Seed Technological Research Unit, MPKV, Rahuri (Maharashtra) India.

Seed germination

Germination test was conducted in three replications of 100 seeds each by adopting between paper methods (ISTA, 2008). Seeds were incubated at slanting position in Walk-in germination room in growth cabinets. The temperature of 25 ± 1°C and RH of 95 per cent was maintained during the germination test (KUCHLAN *et al*., 2010).

Accelerated aging test

Accelerated aging test was conducted by using the jar accelerated aging system. Seeds were placed in muslin cloth bags tied with rubber band and placed on incubator. The accelerated aging test (AAT) was conducted under recommended conditions with slight modification (ISTA, 2008). Fifty grams of soybean seeds of each four genotypes were subjected to an accelerated aging at 100 percent RH and 42°C temperature in an incubator. Individual genotypes were taken in separate petriplate and placed on the wire mesh and incubated (KUCHLAN *et al*., 2010). The samples were drawn after 96 hrs and the germination was tested on three replications of 100 seeds for each genotype (ISTA, 2008).

DNA extraction and molecular characterization

The leaf samples were collected and genomic DNA was extracted based on a previously reported cetyl-trimethyl ammonium bromide (CTAB) method (KEIM *et al*., 1988) with some modification of four genotypes with their F1s & F2s mapping population, and their quality of genomic DNA was checked by using 0.8% (w/v) agarose gel electrophoresis. Polymorphism survey was carried out with 26 SSR markers distributed across the soybean genome. The SSR markers were selected based on the earlier studies and are linked to the seed longevity traits (Table 1).

SSR markers were amplified in a PCR reaction mixture (20µl) contained template DNA (30ng), 2.0µl *Taq* buffer B, MgCl2 (1.7µl), 2.0µl dNTP (2mM) and 1.0µl of forward and reverse primer, and *Taq* DNA polymerase (0.3µl). Amplification was performed in 0.2ml (each tube) thin walled PCR tubes in Ependroff thermal cycler. PCR regime comprised of initial incubation at 94.0°C for 5 min and then subjected to 40 cycles: 94.0°C for 30 sec, 42-56°C for 30 sec and 72.0°C for 30 sec, and final extension was carried out at 72.0°C for 5 min. To check the quality of the extracted DNA, Amplified products were analyzed using 2% metaphor an agarose gel at constant power 80 volts for about 3 hours and gel stained with ethidium bromide, and visualized under UV light.

DNA bulks, two bulks of contrasting phenotypes (high and low) were used for the bulk segregation analysis (ZOU *et al*., 2016; MICHELMORE *et al*., 1991). For formation of 20 contrasting plants each of high and low F2s progeny for seed longevity trait were selected and pooled separately to high seed longevity bulk and low seed longevity bulk. Equal quantities of DNA were bulked from 10 high and 10 low seed longevity F2 progeny to give two DNA bulks for BSA analysis. The one SSR marker (Satt632) in the selected segregating individuals was scored as 0-2 which corresponds to the banding pattern for 'DS-228' (P1), heterozygotes and Birsa Soya1 (P2), respectively.

Table 1. The details of the SSR markers used in the present study

Sr.	QTL	Primers	Sequences	Tann (°C)	Linkage Group	cM	Reference
1.	SCH 1-1	Sat_400	**F**-CGTTGAGCCAATTAAAATTATATTTATGAT	57	A2	49.392	Grant *et al*., 2010
			R-CGCAAATGCCATTCAATTAGTAGTCA				
2.	SCH 1-2	Sat_162	**F**-GCGTGGTTTTTCGCTGGATATA	58		51.862	
			R-GCGCATTTCGTAACATATTTTTCAC				
3.		Satt632	**F**-GGGCTATGAAGGGAATGGAAAGGA	54		51.505	
			R-CCCATATTGAAGATTTGAAGTAAT				
4.		Sat_157	**F**-GCGGTTTTGCAAGATGTGATGAGT	58		51.565	
			R-GCGCGTACGCAAAATTTATATTCA				
5.	SCH 1-6	Satt341	**F**-GCGGAGCTTACCAACATAAAAAAACT	60		77.695	
			R-GCGGTCCAACATTGAGGCAAGAATAC				
6.	RGR-C1	Satt476	**F**-TTTGCTGATTAAAAAAACAAAAACTG	54	C1	80.62	Dargahi et *al.,* 2014
			R-TTGTTAGAATGGGGACTACTTCACTA				
7.		Satt399	**F**-AAGCCAACCTTATAATTCTTTCAT	54		76.23	
			R-ATATGGGCTTACTTACCCATCATAGA				
8.	SCH 1-4	Satt274	**F**-GCGGGGTCAATTAGTTTTCGTCAGTT	60	D1b	116.35	Grant *et al*., 2010
			R-GCGCACGGTATATAATCGAACCTAT				
9.		Sat_202	**F**-CG GGC CAA GTG AAT AAC AAG TTT ATA AAT T	60		118.95	
			R-CG TCC TGC CAC ACC ATT TTT ATA CTT TTA A				
10.	SH 1-2	Satt531	**F**-GCATGCAACTGAGGGAGCAGAT	53	-	40.87	Grant *et al*., 2010
			R-GCCACAAATTATGCAGAATATA				
11.		Satt184	**F**-GCGCTATGTAGATTATCCAAATTACGC	52		17.52	
			R-GCCACTTACTGTTACTCAT				
12.	RGR-F	Satt269	**F**-GCGTGCCAGGTAGAAAAATATTAG	56	F	11.37	Dargahi et *al.,* 2014
			R-GCGGTTTTTCACTTTTCAAAATTC				
13.		Satt423	**F**-TTCGCTTGGGTTCAGTTACTT	49		20.56	
			R-GTTGGGGAATTAAAAAAATG				
14.	SCH 1-3 /SH 1-1	Sat_286	**F**-CGTTGCTTGCTAAGTAGTGTTTTTAATCCT	60	L	87.42	Grant *et al*., 2010
			R-CGTCTCCCATCATGCAACTTCAATA				
15.		Satt664	**F**-GCGTAGATGCTCAACATCAACACTAATCTG	63		92.66	
			R-GCGGACGATGAAGAAATATACTATTACGAA				
16.		Satt229	**F**-TGGCAGCACACCTGCTAAGGGAATAAA	60		93.89	
			R-GCGAGGTGGTCTAAAATTATTACCTAT				
17.	RGR-L	Satt523	**F**-GCGATTTCTTCCTTGAAGAATTTTCTG	60		27.92	Dargahi et *al.,* 2014
			R-GCGCTTTTTCGGCTGTTATTTTTAACT				
18.		Satt143	**F**-GTGCCACAAATTTAAAATTACTCA	54		30.19	
			R- TCCCTCCCTTTTGATTTACAC				
19.	SCH 1-5	Satt530	F-CATGCATATTGACTTCATTATT	51	N	32.85	Grant *et al*., 2010
			R-CCAAGCGGGTGAAGAGGTTTTT				
20.		Satt641	**F**-GCGGAACATCACGGTTATA	54		29.28	
			R-GCGGGAGGCTCTGTCTCTTAGA				
21.	SCH 1-7	Sat_379	**F**-GCGTTTTGGCTCATCTTTCTTTTA	58		4.33	Grant *et al*., 2010
			R-GCGGCCCTAAGCACAACTGAACCTAT				
22.	Seed colour and durability markers	Satt371	**F**-TGCAAACTAACTGGATTCACTCA	54	-	-	Hosamani *et al*., 2013
			R-GAGATCCCGAAATTTTAGTGTAACA				
23.		Sat453	**F**-GCGGAAAAAAAACAATAAACAACA	54		-	
			R-TAGTGGGGAAGGGAAGTTACC				
24.		Satt618	**F**-GCGGTGATATTACCCCAAAAAAATGAA	60		-	
			R-CGCTAGTTTCTAGTGGAAAGATGAGT				
25.	-	Satt285	**F**-GCGACATATTGCATTAAAAACATACTT	57		-	Singh *et al*., 2008
			R-GCGGACTAATTCTATTTTACACCAACAAC				
26.		Satt460	**F**-GCGCGATGGGCTGTTGGTTTTTAT	60		-	
			R-GCGCATACGATTTGGCATTTTTCTATTG				

SCH-Seed Coat Hardiness; SH- Seed Hardiness; qRGR- Relative Germination Rate

Data analysis

Segregation of each marker in the mapping population was tested for goodness of fit to an expected 1:2:1 ratio using the *chi-square* (X^2) analysis. Genotyping of the F2 population was carried out using markers which showed polymorphisms. Associations of marker loci with QTLs were considered significant when the level of significance was 0.05 (Singh, 2008). Candidate i*n silico* genes for seed longevity within the fine mapped Satt632 region available in the Phytozome V9.1 genome database (http://www.phytozome.net/cgi-bin/gbrowse/soybean/). The genomic region linked with Satt632 region was browsed for open reading frames/transcripts present in between 8.0 and 8.6 million base pair region of chromosome 08**.** SSR markers were synthesized following the sequences published in the SoyBase website http://www.soybase.org/sbt/search/search/results.php?category=LocusName&earch_term).

RESULTS

The present investigation was undertaken for the molecular tagging of high seed longevity markers. Initially the four genotypes (Kalitur, Birsa Soya1, DS-228 & MAUS-71) were investigated for polymorphism to a shortlist 26 SSR primer pairs known to amplify markers linked to the storability and shortlisted primers were used for the bulk segregant analysis. For a bulk segregant analysis high and low seed longevity bulks were derived from DNA of 20 F2s segregants each of having highest (84 to 92%) and lowest (44 to 56%) seed longevity. Individual F2 segregants of the four crosses undertaken exhibited 40 to 92% (Table 2.) vigour on accelerated aging with an almost same mean vigour value (71.61 to 74.33%).

Table 2. Variation of accelerated aging test and ambient germination in soybean

Crosses	Generations	% Germination (ambient conditions)	% Germination (accelerated aging)
DS-228 x Kalitur	P_1	69.33	54.26
	P_2	95.33	82.55
	F_2	84.67	72.72 (48 to 84)
DS-228 x Birsa Soya1	P_1	70.67	53.60
	P_2	90.67	82.40
	F_2	82.67	71.61 (44 to 92)
MAUS-71 x Birsa Soya1	P_1	70.67	54.54
	P_2	88.67	81.12
	F_2	82.67	73.58 (40 to 92)
MAUS-71 x Kalitur	P_1	69.33	57.17
	P_2	96.67	82.89
	F_2	87.33	74.33 (52 to 92)

SSR analysis

Among the 26 SSR markers used, 06 markers were polymorphic (Sat_162, Satt632, Sat_202, Satt523, Satt184 and Satt460), it showed a polymorphism between genotypes differing for seed longevity i.e. two high seed longevity (Birsa Soya1, Kalitur) and two low seed longevity genotypes (DS-228 & MAUS-71) that were used for the developing F2s segregating population.

Hence were included in further BSA studies (Table 3.) and rest of the 20 primers yielded either monomorphism or banding sometimes shared by genotypes with contrasting trait making them non-informative and therefore were excluded from further analysis (Fig.1). Satt632 showed an polymorphism corresponding with contrasting seed longevity trait *i.e.* Satt632-237 bp marker for QTL *SCH 1-2* got amplified in both high seed longevity genotypes (Birsa Soya1 and Kalitur); while a 246 bp marker in both low seed longevity genotypes (DS-228 and MAUS-71). QTL *SCH 1-2* specific Sat_162-152 bp and Sat_162-168 bp marker got amplified in high seed longevity genotypes Birsa Soya-1 and Kalitur, respectively; while a Sat_162-176 bp marker got an amplified in both low seed longevity genotypes (DS-228, MAUS-71). In the Sat_202 known to the amplify markers specific QTL *SCH 1-4*, amplified a 321 bp marker in both high seed longevity genotypes (Birsa Soya1, Kalitur) while 312 bp and 309 bp markers got an amplified in DS-228 and MAUS-71, respectively. The seed hardiness loci *qSH1-2* linked Satt184-194 bp marker amplified only in the both high seed longevity genotypes (Birsa Soya1 and Kalitur); while an 144 bp marker with amplified in both low seed longevity genotypes (DS-228 and MAUS-71). Satt523 primer, known to amplify markers linked to QTL *RGR-L*, amplified 175 bp (Birsa Soya1), 185 bp (Kalitur) and 185 bp markers in the both low seed longevity genotypes (DS-228, MAUS-71). The seed coat permeability specific Satt460- 112 bp marker got amplified in the both high seed longevity genotypes (Birsa Soya1 and Kalitur) while a 118 bp marker was amplified in both low seed longevity genotypes (DS-228 and MAUS-71). According to these results, six primers were amplified markers and linked to four QTLs *viz.*, *qSCH 1-2*, *qSCH 1-4*, *qSH 1-2* and *qRGR-L* (Fig.1).

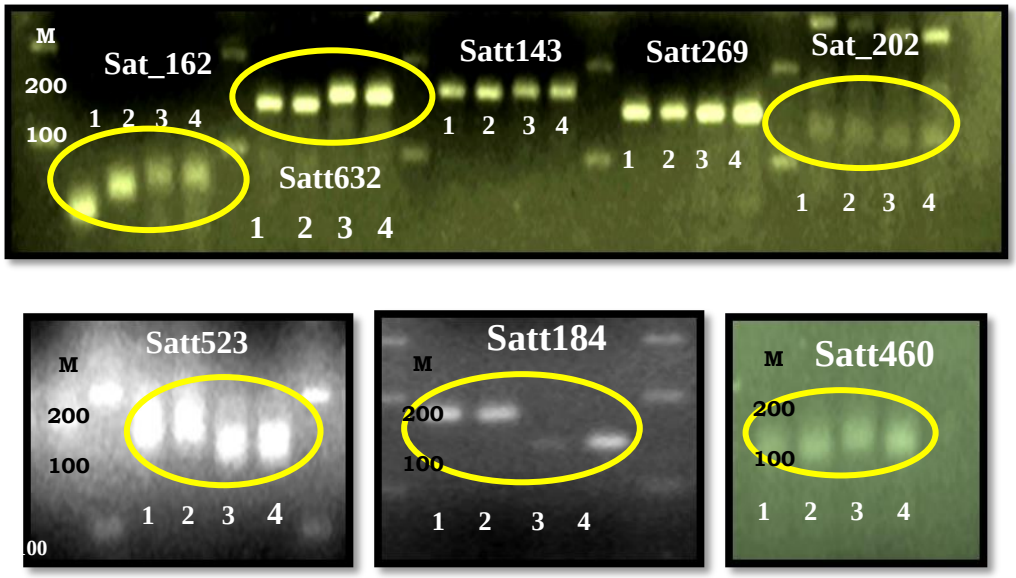

Fig 1. SSR markers which showed polymorphism between parental lines

M: 100 bp Ladder; **1**-Birsa Soya-1; **2**-Kalitur; **3**- DS-228; **4**-MAUS-71

Table 3. SSR markers which showed polymorphism in between parental lines

Primers	Size range (bp)	Chromosome	QTL	High seed longevity		Low seed longevity	
				Bisa Soya-1	Kalitur	DS-228	MAUS-71
Sat_162	152 to 176	A2	qSCH 1-2	152	168	176	176
Satt632	237/246			237	237	246	246
Sat_202	309/321	D1b	qSCH 1-4	321	321	312	309
Satt523	163 to 185	L	qRGR-L	175	185	163	163
Satt 184	144/194	F	qSH 1-2	194	194	144	144
Satt460	112/118	--	qSCP	112	112	118	118

Bulk Segregant Analysis

Bulk segregant analysis carried out with the 06 shortlisted SSR primers for tagging of markers for seed longevity loci in soybean, among them, Sat_162, Satt632, Sat_202, Satt523, Satt184 and Satt460 were polymorphic between genotypes (Birsa Soya1 & DS-228), but failed to show matching polymorphism in between their bulks (high and low), hence they were excluded from individual F2 plants study (Fig.2). SSR primer (Satt632) produced a 237 bp marker both in the high seed longevity genotype as well as bulk (HB); while a Satt632-246 bp allele got amplified in low seed longevity genotype and bulk (LB). This indicates that its likely candidature for seed longevity marker as it is also known to be linked to seed coat hardiness loci *qSCH 1-2* (Fig.2).

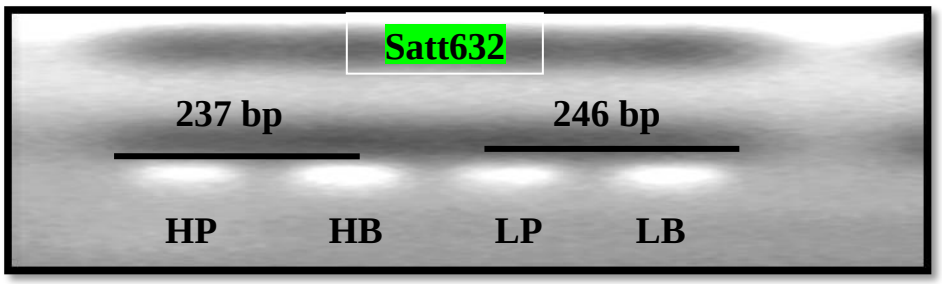

Fig 2. BSA for seed longevity trait in the crosses DS-228 x Birsa Soya1 using SSR markers
M: 100 bp Ladder; **HP**-High seed longevity genotype; **HB**-High seed longevity bulk;
LP-Low seed longevity genotype; **LB**-Low seed longevity bulk

Validation of Satt632-*237 bp marker qSCH 1-2 in individual F2 populations*

SSR primer Satt632 identified on bulk segregant analysis was used for validation analysis of 93 individuals in segregating F2 population (from Birsa Soya1 × DS-228) which were contrasting for seed longevity trait. The association of markers with the soybean high seed longevity trait was analyzed in F2 population as well as control DS-228 and Birsa Soya1 genotype. Satt632-237 bp marker got amplified in 73 F2 plants (Birsa Soya-1 × DS-228); Satt632-246 bp allele marker got amplified in 20 F2 plants; while no heterozygotes were

observed (Fig. 2). Satt632-237 bp allele amplified only in the high seed longevity F2 plants, whereas a Satt632-246 bp allele was present only in low seed longevity plants. Hence, the primer Satt632 is impending marker for marker assisted breeding for high seed longevity for *qSCH1-2* like gene present on chromosome A2. The validated primers on various segregating plant populations are much more useful in marker assisted breeding. The *chi-square* analysis of Satt632 primer in F2 plants showed non-significant *chi-square* values, Satt632 0.05 (P=0.81) for cross DS-228 x Birsa Soya1 indicating that soybean seed longevity trait is governed by one or few gene with observed segregation ratio of 3.65:1 as against expected segregated ratio 3:1 (Table 4).

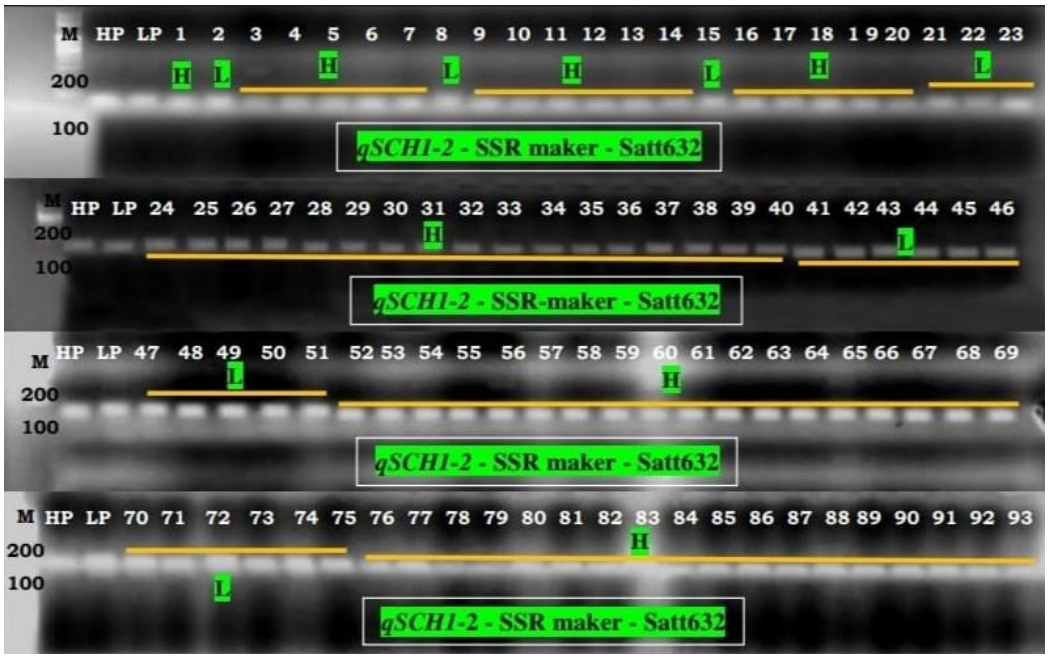

Fig 3. Validation of SSR marker Satt632 (*qSCH 1-2*) in individual F_2 progenies: **M:** 100 bp Ladder; **HP**-High seed longevity gentype (Birsa Soya1), **LP**-Low seed longevity genotype (DS-228), **F2 progenies**- 1 to 93

Table 4. Validation of F_2 plants of cross Birsa Soya-1 × DS-228 for Satt632 (SCH 1-2)

Traits	Observed plants	Expected Plants	Expected ratio	X^2 value	X^2 table	Probability
High seed longevity	73	69.75	3:1	0.05	3.84	0.81
Low seed longevity	20	23.25				

Total	93	93

Figures in the parentheses indicate recombinants

Table 5. Phytozome analysis of Glycine max chromosome 08 Satt 632 marker region

Location	Name	ORF Id
Gm08:8114056..8126761	agamous-like MADS-box protein AGL3-like	HQ636609.1
Gm08:8182656..8185890	small nuclear ribonucleoprotein	Glyma.08g105900
Gm08:8189613..8192578	Amidase	BT094288.1
Gm08:8208987..8215162	Pentatricopeptide repeat (PPR)	Glyma.08g106500
Gm08:8216338..8219979	peptidyl-prolyl-cis-trans-isomerase	Glyma.08g106700
Gm08:8221055..8223572	Mitochondrial/chloroplast ribosomal protein S19	Glyma.08g106800
Gm08:8223512..8223774	Satt 632 marker region	
Gm08:8229694..8231371	CGI-12 PROTEIN-RELATED (Mitochondrial transcription termination factor family protein)	Glyma.08g107000
Gm08:8242346..8245459	Tic22-like family proteins	Glyma.08g107200
Gm08:8250679..8253218	brassinosteroid-regulated protein	BT096944.1
Gm08:8266733..8268742	Glucosyl/Glucuronosyl Transferases (UDP-glucuronosyl and UDP-glucosyl transferase)	Glyma.08g107500
Gm08:8284887..8288364	RHG4 like receptor kinase	DQ439710.1
Gm08:8358422..8362950	serine hydroxymethyltransferase 2	EU912420.1
Gm08:8365028..8370987	SUB1 substilin like protease homolog	JQ762395.1
Gm08:8388811..8390099	chalcone synthase	FJ770471.1
Gm08:8476031..8477319		FJ770471.1
Gm08:8536017..8543555	Lectin	FJ501173.1
Gm08:8559486..8562813	geranylgeranyl transferase type-2 subunit beta-like	BT093796.1

In silico candidate gene analysis

An attempt was made to the search probable candidate genes for seed longevity within the fine mapped Satt632 region available in the Phytozome V9.1 genome database (http://www.phytozome.net/cgi-bin/gbrowse/soybean/). Candidate gene analysis, Satt632 marker flanking site within 8.2-8.4 million base pair region of chromosome 08 regions was found to have a few genes. Of them genes encoding either for a brassinosteriod regulated protein and Pentatricopeptide repeat (PPR) appear to be the most likely candidate gene for seed longevity. The different genes present in this region encoding for different proteins are given in (Table 5) along with their locations. In addition, adjoining region further between 8.4-8.6 million base pair region was found to have genes encoding for chalcone synthase; lectin; geranyl geranyl transferase type-2 subunit.

DISCUSSION

Investigation on seed longevity trait in soybean genotypes to identify parental polymorphism to a shortlist 26 SSR primer pairs known to amplify markers linked to the

storability and shortlisted primers were used for the bulk segregant analysis. For a bulk segregant analysis high and low seed longevity bulks were derived from DNA of 20 F2s segregants each of having highest and lowest seed longevity. Hence were included in further BSA studies (Table 3) and rest of the 20 primers yielded either monomorphism or banding sometimes shared by genotypes with contrasting trait making them non-informative and therefore were excluded from further analysis (Fig.1). Satt632 showed an polymorphism corresponding with contrasting seed longevity trait *i.e.* Satt632-237 bp marker for QTL *SCH 1-2* got amplified in both high seed longevity genotypes (Birsa Soya1 and Kalitur); while a 246 bp marker in both low seed longevity genotypes (DS-228 and MAUS-71). The seed hardiness loci *qSH1-2* linked Satt184-194 bp marker amplified only in the both high seed longevity genotypes (Birsa Soya1 and Kalitur); while an 144 bp marker with amplified in both low seed longevity genotypes (DS-228 and MAUS-71). In tropical conditions seed coat hardiness is a quantitative trait that contributes to viability of stored seeds (Potts et al. 1978; Keim et al. 1990). The strength of the seed coat provides protection from mechanical damage during with pre-harvest and post-harvest operations (KUCHLAN et al., 2010) According to these finding, six primers were amplified markers and linked to four QTLs *viz.*, *qSCH 1-2*, *qSCH 1-4*, *qSH 1-2* and *qRGR-L* (Fig. 1). Identified few SSR markers included Satt184 (*qSH1-2*) and Satt460 (*qSCP*) which produced high/low specific allelic making them candidate markers for linkage with seed storability (HOSAMANI *et al*., 2013). In correlation analysis the presence of Satt632-237bp marker had highly positive significant with high seed longevity. As per SOYBASE website (GRANT *et al*., 2010), Satt632 amplified markers is linked to seed coat hardiness loci *qSCH 1-2*.

SSR primer (Satt632) produced a 237 bp marker both in the high seed longevity genotype as well as bulk (HB); while a Satt632-246 bp allele got amplified in low seed longevity genotype and bulk (LB). This indicates that its likely candidature for seed longevity marker as it is also known to be linked to seed coat hardiness loci *qSCH 1-2* (Fig.2). Hence, the primer Satt632 is impending marker for marker assisted breeding for high seed longevity for *qSCH1-2* like gene present on chromosome A2. Previously there are reports of identification of markers (from different linkage groups) associated with seed storability (SAKAMOTO *et al*., 2004; SINGH *et al*., 2008; DARGAHI *et al*., 2014). The validated primers on various segregating plant populations are much more useful in marker assisted breeding.

Soybean seed longevity trait is governed by one or few gene with observed segregation ratio of 3.65:1 as against expected segregated ratio 3:1 (Table 4). Candidate gene analysis, Satt632 marker flanking site within 8.2-8.4 million base pair region of chromosome 08 regions was found to have a few genes. Of them genes encoding either for a brassinosteriod regulated protein and pentatricopeptide repeat (PPR) (BAKAN and SMALL, 2014; MANAVSKI *et al*., 2012; BRYANT *et al*., 2011; DING *et al*., 2006; LEUBNER *et al*., 2001; CLOUSE *et al*., 1998) appear to be the most likely candidate gene for seed longevity.

CONCLUSIONS

In the present study, identify a set of good storer soybean breeding lines along with candidate SSR markers for identifying markers linked to seed storability traits, the primer Satt632 can be utilized for validation in the crosses between DS-228 × Birsa Soya1 and their F2 population to determine QTLs controlling the trait and also for breeding commercial lines. There is a need of more number of markers need to be evaluated to get complete expression of seed

longevity and for identifying QTLs. Hence, the primer Satt632 is impending marker for marker assisted breeding for linked to candidate gene analysis of seed coat hardiness loci *qSCH1-2* is present on chromosome A2, it indicated for the role of either a brassinosteriod regulated protein or pentatricopeptide repeat gene to regulate high seed longevity in soybean. Seed aging may be associated with deteriorative changes in membranes. Accelerated aging test can be used as simple and easy but indirect measure of seed longevity in soybean. Thus, the accelerated ageing test can be used to predict the relative storability in soybean genotype based on seed germination. The yellow seeded variety with low longevity and black seeded variety with higher longevity had been crossed, and seed coat hardiness loci *qSCH1-2* QTLs controlling the trait in the breeding lines will be used for improvement of seed storability in soybean and also to develop future RILs mapping population for seed longevity-related traits.

ACKNOWLEDGEMENTS

The authors are grateful to establishment of Mahatma Phule Krishi Vidyapeeth, Rahuri and the office In charge of State Level Biotechnology Centre, MPKV, Rahuri for providing necessary facilities to undertake this study. The author is also thankful to the Indian Council of Agricultural Research (ICAR), New Delhi for financial support under ICAR-SRF (PGS) with fellowship in the discipline of Genetics and Plant Breeding.

Received, October 18th, 2022
Accepted May 18th, 2023

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MOLEKULARNA KARAKTERIZACIJA DUGOVEĆNOSTI SEMENA SOJE [*Glycine max* (L.) Merrill] KORIŠĆENJEM SSR MARKERA

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Izvod

Soja je najvažnija uljarica u tropskim i suptropskim delovima sveta, nažalost, nema dovoljno sposobnosti za dugovečnost semena. Na njega snažno utiče okruženje (temperatura i relativna vlažnost) tokom formiranja semena, žetve i skladištenja. Označavanje markera vezanih za dugovečnost semena u ukrštanjima (Birsa Soia1 × DS-228) koji su genotipovi dobri i loši za skladištenje, urađeno je korišćenjem 26 SSR prajmera. Polimorfizam je primećen između genotipova koji se razlikuju po osobinama dugovečnosti semena za šest parova prajmera, tj. Sat_162, Satt632, Sat_202, Satt523, Satt184 i Satt460, među njima QTL za otpornost omotača semena, *SCH* 1-2 povezani sa Satt632 amplifikovali su različite markere. Grupna segregantna analiza je sprovedena korišćenjem SSR markera u segregaciji 93 F2 populacije. Satt632-237 bp marker je dalje validiran u pojedinačnoj populaciji biljaka F2. Utvrđeno je da su genotip Birsa Soya1 i marker Satt632-237 bp usko povezani sa dugotrajnošću semena soje. Dakle, prajmer Satt632 je marker za oplemenjivanje uz pomoć markera, povezan sa dugovečnošću semena i otpornosti omotača semena na lokusu *qSCH1-2* koji je prisutan na hromozomu A2. Svojstvo dugovečnosti semena je regulisano jednim ili sa nekoliko gena sa posmatranim odnosom segregacije od 3,65:1 u odnosu na očekivani odnos segregacije 3:1. Analiza gena kandidata na *qSCH1-2* lokusu, ukazuje na ulogu ili proteina regulisanog brasinosteriodom ili pentatrikopeptida, značajnog za regulisanje dugovečnosti semena.

Primljeno 18.X.2022.
Odobreno 18. V 2023.