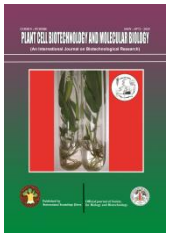

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Identification of Soybean (*Glycine max* L.) Segregants for Kunitz Trypsin Inhibitor and Lipoxygenase Free Gene Using Linked Molecular Markers

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The presence of anti-nutritional factors such as *Kunitz trypsin inhibitor* and enzymes lipoxygenase, which contributes to the beany off-flavor, are key factors limiting consumer acceptance of soybeans. Kunitz trypsin inhibitor binds to and inhibits the activity of proteases (trypsin) found in the digestive systems of most mammals. The beany flavor in soybeans arises from the breakdown of PUFAs by lipoxygenase into hydroperoxide derivatives, which break down into various volatile compounds, creating the off-flavor. This undesirable flavor in soy products is linked to activity of

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lipoxygenase-1, lipoxygenase-2, and lipoxygenase-3 in soybean seeds, which are regulated by the dominant genes *Lox1*, *Lox2*, and *Lox3*, respectively. The current study involved molecular evaluation in the 21 F_1s and segregating generation (31 F_2s and 59 F_3s) plants of three crosses *viz*., MACS 1520 × NRC 142, KDS 1201 × NRC 153 and KDS 1201 × TGX 709-50E. Total one hundred eleven plants from three crosses were analysed to check for dysfunctional alleles of *KTI, LOX-2* and *LOX*-3 genes*.* The final findings showed that two plants out of them were triple null *i.e*. they do not have functional *Kunitz trypsin inhibitor* and *Lipoxygenase 2* and *Lipoxygenase 3* genes. Hence these two plants were absolutely promising for development of cultivar which triple null soybean. Six more plants were double null *i.e.* free from both *KTI* and *LOX-2*, therefore they can be used in homozygous genotype development in soybean breeding. It can lead to the attending the problem of soybean full protein utilization by human and ensures soybean long term storage.

Keywords: Molecular breeding; segregants; double null; triple null.

1. INTRODUCTION

Soybean (*Glycine max* L.) is an annual dicotyledonous plant belonging to the family of Fabaceae (Leguminosae) commonly known as golden bean or miracle bean or soja bean. Soybean seeds are being packed with basic nutrients like protein, essential amino acids, fats, polyunsaturated fatty acids, minerals and vitamins have the potential to combat the malnutrition in developing countries (Kumar et al*.,* 2001). The protein and oil account for 60% (high protein 40% and oil 20%) of the seed with about 30% carbohydrates. In addition, 100 g soybean contains 240 mg calcium, 690 mg phosphorus, 11.5 mg iron, 432 calories, 10.5 g fats and 426 mg of vitamins (A, B and D) (Nagraj, 1995).

Despite this, the presence of a high concentration of anti-nutritional factor (trypsin inhibitor) in soybean seed limits protein digestion. Kunitz factor is trypsin inhibitors present in raw soybeans that block protease enzymes in the digestive system. The reduce trypsin activity (a protease enzyme secreted by the pancreas) and impair protein digestion by monogastric animals and some young ruminant animals. The molecular mass of the Kunitz trypsin inhibitor (KTI) is 20.1 kDa, with a single peptide chain of 170 - 200 amino acids (Steiner, 1965; Koide and Ikenaka, 1973). It is not suggested to give raw soybeans to monogastric species such as chickens and pigs since the presence of trypsin inhibitors and lectins would result in stunted growth, poor feed efficiency, and pancreatic hypertrophy. KTI is primarily responsible for majority of trypsin inhibitor activity (Pesic et al*.,* 2007). This antinutrient is heat labile, however the heat inactivation procedure, which is carried out by boiling (20 minutes), not only incurs additional costs for processors, but it also results in the insolubilization of valuable soy-proteins (Rani and Kumar, 2015). The elimination or nullifying the KTI effect is one of the major objectives of research.

The undesired off-flavor created in soy products during processing is one of the biggest limits to their usage in food. The beany flavour in soybean results from the degradation of PUFAs by lipoxygenase (LOX) into hydroperoxyl derivatives, which further break down into different volatile compounds, resulting in the beany flavor (Wang et al*.,* 2021). This undesirable flavor in soy products is linked to activity of lipoxygenase-1, lipoxygenase-2, and lipoxygenase-3 in soybean seeds, which are regulated by the dominant genes *Lox1*, *Lox2*, and *Lox3*, respectively (Rani and Kumar, 2015). Heat inactivation of lipoxygenases at the industrial level is not only inefficient, but it also has an impact on protein functionality. As a result, genetic eradication of lipoxygenase-2 is critical to increasing soybean consumption in food applications. Lipoxygenase-2 is the primary producer of n-hexanal among the three lipoxygenases. *Lipoxygenase-2* genetic deletion has been shown to improve the flavor of soy products (Rawal et al*.,* 2020).

In the current study, soybean genotypes free from both of these undesirable components are developed by introgression of null alleles of *KTI, Lox2* and *Lox3*. It is desirable to have soybean varieties genetically free from Kunitz trypsin inhibitor (KTI) and lipoxygenases (Lox) in order to increase consumption and address the need of soy food producers the soybean that are free of the lipoxygenase gene and the Kunitz Trypsin Inhibitor *i.e.,* double null are developed.

Keeping in view the above facts, the present research work was carried out to study the

molecular evaluation of soybean genotypes and segregants for null *kti* and *lox* alleles for the genetic elimination of this undesirable component.

2. MATERIALS AND METHODS

The present investigation was carried out at State Level Biotechnology Centre and Post Graduate Farm, Mahatma Phule Krishi Vidyapeeth, Rahuri. The material used in present investigation was derived from the crosses (Table 1) developed by two Ph.D. students (Bhagyashree Bhosale, unpublished; Punam Gharge, Unpublished) in which soybean genotypes that are free of the *Kunitz Trypsin Inhibitor* as well as the *lipoxygenase* gene are used. Which are developed by ICAR-Indian Institute of soybean Research, Indore *i.e.* NRC 142 and NRC153. KDS 1201 was developed by MPKV, Rahuri at Agriculture Research Station Kasbe Digraj, Sangli, Maharashtra.

The various generations like F_1, F_2 and F_3 with their numbers are as depicted in Table 1. The genomic DNA was extracted from tender leaves from one hundred eleven plants of respective generation following the cetyl trimethyl ammonium bromide (CTAB) genomic DNA extraction method with some modifications described by Doyle and Doyle (1990). Isolated genomic DNA samples were resolved on 0.8% (w/v) agarose by agarose gel electrophoresis for quality checking and analysed by Nanodrop for quantitative estimation to check DNA quality and quantity. The agarose gel electrophoresis and NanoDrop spectrophotometer confirmed the purity and integrity of genomic DNA.

SSR and gene specific primers were used for assessing parental polymorphism and progeny and details given (Table 2). These markers are reported to be tightly linked to *KTI*, *LOX* null alleles by earlier research works (Kumar et al., 2013; Jin et al., 2012; Kumar et al., 2014; Reinprecht et al., 2011) therefore used for screening of *Kunitz trypsin inhibitor*, *lipoxygenase 2*, *lipoxygenase 3* free soybean plants.

PCR amplification reaction mixtures of 15µl were prepared in sterile 0.2 ml PCR tubes containing the components in Taq buffer B (without $MgCl_2$) 1.5µl(10X), $MgCl_2$1.2µl (25mM), dNTPs mix1.5µl (10mM), primer both forward and reversed 0.75µl each (20 picomoles), *Taq* DNA polymerase 0.33µl (3unit/µl), DNA 2µl (30-50ng/µl) proportion and rest of double distilled water (6.97µl) to makeup 15µl reaction master mix. Then, PCR tubes were loaded in a thermal cycler and the requirement of time, temperature and cycles were carefully run. The initial denaturation at 94°C for 4 min, denaturation at 94°C for 45 second annealing temperature is differ primer wise for 1 min. The extension is at 72°C for 1 min. and final extension at 72°C for 10min. the annealing temperature of *titi* specific, *Satt 656*, *lx2* specific, and *Lox-3-3'* is 51°C, 51°C, 53°C and 53°C respectively.

To check PCR amplification profile, PCR amplified DNA from marker sample was resolved in 3% (*Satt 656* primer) and 2.5% (gene specific*lx2*, gene specific *titi* and *Lox-3-3'* primers) agarose gel. Voltage was set at 75V and DNA samples were run for 1.5-2 hrs, until PCR amplicons resolved clearly.

3. RESULTS AND DISCUSSION

In the current investigation, genomic DNA of one hundred eleven soybean plants (21 F_1s, 31 F_2s and 59 F_3s) of three crosses *viz*., MACS 1520 × NRC 142, KDS 1201 × NRC 153 and KDS 1201 × TGX 709-50E were analysed with primers specific to null allele of *KTI, Lox2* and *Lox3* genes, results of which are discussed below:

Table 1. Crosses and plants taken from each generation for molecular assay

Sr. No.	Cross	F_1	F_2	F_3	Total
1.	MACS 1520 × NRC 142 (High Yielder) (null *kti+lox2*)	21	-	27	48
2.	KDS 1201 × NRC 153 (*kti* free) (null *lox2* + *lox3*)	-	13	14	27
3.	KDS 1201 × TGX 709-50E (*kti* free) (Delayed wilting)	-	18	18	36
	Grand Total				111

Table 2. Details of primers used in present study

Sr. No.	Name of Primer	Markers Sequence (5'-3')	Reference
KTI-3 gene			
1.	Gene *titi* specific	F: CTTTTGTGCCTTCACCACCT R: GAATTCATCATCAGAAACTCTA	Kumar et al., 2013
LOX-2 gene			
2.	lx_2 specific	F: AAACCAGTAAGATAACAGCAGATG R: AATGGCTCAATCACCGCT	Jin et al., 2012
3.	*Satt 656*	F: GCGTACTAAAAATGGCAATTATTTGTTG R: GCGTGTTTCAGTATTTGGATAATAGAAT	Kumar et al., 2014
LOX-3 gene			
4.	*Lox-3-3'* (STS)	F: CGGCTTGATAACCCATTGTT R: CAAGCATTGTCCCAAACTTC	Reinprecht et al., 2011

3.1 Molecular Markers-Based Analysis of *Kunitz trypsin inhibitor* Gene in Soybean

In the current study *KTI* gene was studied in segregants from (MACS 1520 × NRC 142, KDS 1201 × NRC 153 and KDS 1201 × TGX 709-50E) crosses using gene specific (*titi*) primers. Gene specific *titi* primer used in this study, yielded no amplicon in Kunitz trypsin inhibitor expressing parents *i.e.* MACS 1520, NRC 153 and TGX 709-50E; while an amplicon of 420 bp size was observed in Kunitz trypsin inhibitor free parents *i.e.* NRC 142 and KDS 1201. Subsequently the F_1, F_2 and F3 segregants along with parents amplified with same primer (Gene specific *titi*)

3.1.1 MACS 1520 × NRC 142

The cross first constitute MACS 1520 (F) and NRC 142 (M) in which NRC 142 free from Kunitz trypsin inhibitor. For this cross the molecular analysis of both parents, F_1 and F_3 generation. A total 48 plant derived from cross MACS 1520 × NRC 142 were studied with gene specific *titi* primer; among that 21 plants were F_1 while remaining 27 plants were of F_3 generation. The molecular analysis of 21 F_1 plants revealed that the amplicon at 420 bp allele in 7 F_1 plants (Plate 1). The plants #1, #4, #5, #9, #11, #13, #17 were free from *KTI* hence can be grown further for KTI free varietal development.

Similarly, the molecular pattern analysis of 27 F_3 generation showed that the 11 of the, viz., #24, #25, #27, #28, #30, #32, #34, #35, #38, #46 and #47, were had the null kti allele by showing 420 bp amplicon of male parent (Plate 2).

3.1.2 KDS 1201 × NRC 153

The cross constitute parent KDS 1201 which free from KTI used as female, while NRC 153 (free for *lox* gene) used as male which do not free from KTI. Hence molecular analysis with *titi* specific gene shows amplicon of 420 bp in only KDS 1201 parent. The two subsequent generation of this cross *viz*., F_2 and F_3 were studied for same primer. The total 27 plants were studied in which 13 plants are of F_2 and 14 are of F_3 generation. The F_2 plants analysis revealed that among 13 plants only 11 plants *i.e*. #1, #2, #3, #4, #5, #6, #8, #9, #10, #11 and #13 have 420 bp amplicon which is in female parent *i.e.* free from KTI (Plate 3).

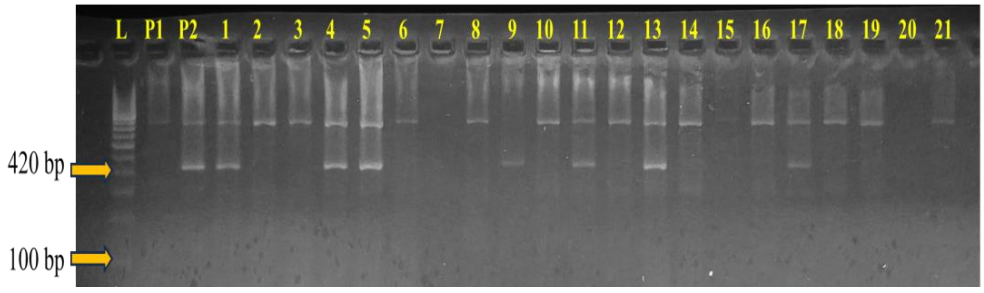

Plate 1. PCR amplification pattern observed in MACS 1520 (P_1) × NRC 142 (P_2), in 21 F_1 plants using gene specific *titi* primer, L- StepUp 100 bp DNA ladder

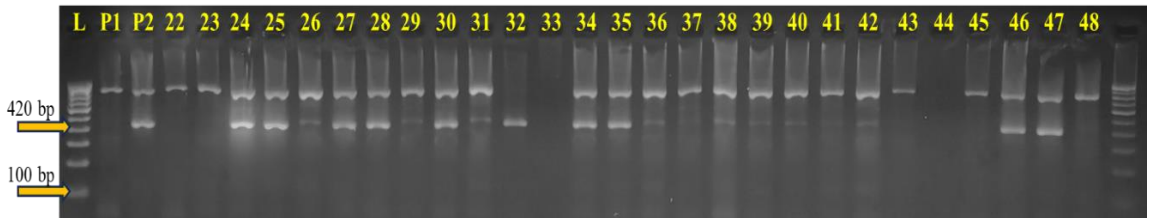

Plate 2. PCR amplification pattern observed in MACS 1520 (P_1) × NRC 142 (P_2), in 27 F_3 plants using gene specific *titi* primer, L- StepUp 100 bp DNA ladder

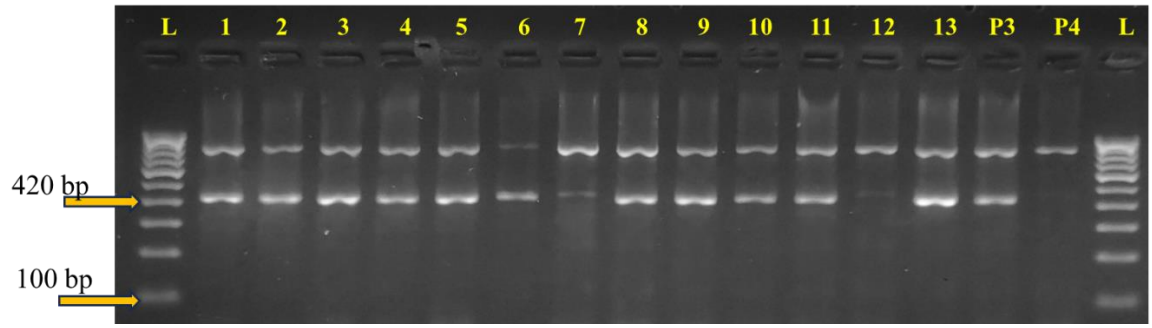

Plate 3. PCR amplification pattern observed in KDS 1201 (P_3) × NRC 153 (P_4), in 13 F_2 plants using gene specific *titi* primer, L- StepUp 100 bp DNA ladder

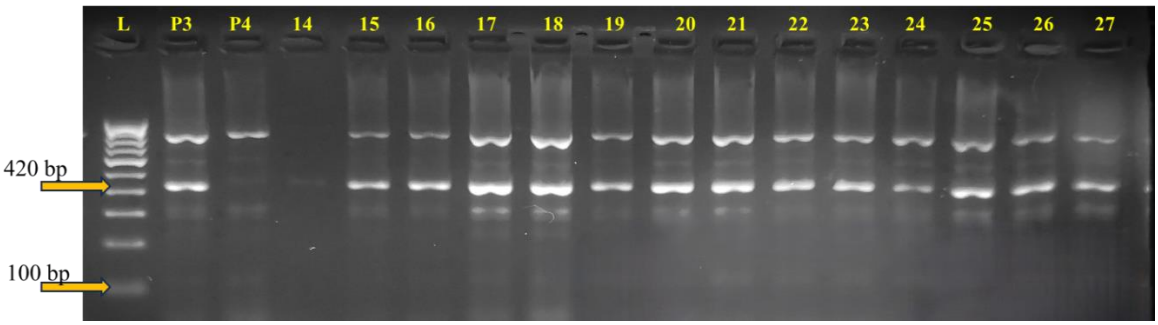

Plate 4. PCR amplification pattern observed in KDS 1201 (P_3) × NRC 153 (P_4), in 14 F_3 plants using gene specific *titi* primer, L- StepUp 100 bp DNA ladder

In the same extent the 14 F_3 plants of same cross KDS 1201 (P_3) × NRC 153 (P_4) were characterized using *titi* primer which showed that 13 plants *viz.,* #15, #16, #17, #18, #19, #20, #21, #22, #23, #24, #25, #26 and #27 were positive for the *titi* amplicon at 420 bp (Plate 4).

3.1.3 KDS 1201 × TGX 709-50E

The third cross constitutes KDS 1201 (P_5) as a female and TGX 709-50E (P_6) as male parent. As stated earlier the KDS 1201 is free from KTI hence amplified band at 420 bp. The analysis involved 36 plants of segregating generation in which 18 plants of F_2 and 18 plants of F_3 generation.

Among 18 F_2 plant studied the 11 plants *viz*., #1, #2, #3, #4, #5, #7, #10, #12, #13, #17 and #18 shows the presence of *titi* allele so can be further advanced to generate KTI free genotypes (Plate 5). The molecular studied with 18 F_3 plants with *titi* specific primer revealed that the 7 (#20, #22, #27, #29, #30, #33 and #36) plants carrying *ti* allele for null KTI (Plate 6). so can be further advanced to generate KTI free genotypes.

In all *titi* specific amplification, the common monomorphic band of 880 bp were also observed which was early detected by Pawale et al*.,* (2021) and was also reported similar result for *titi* specific primer. The Rani et al., (2020) also recorded equivalent result for *titi* primer.

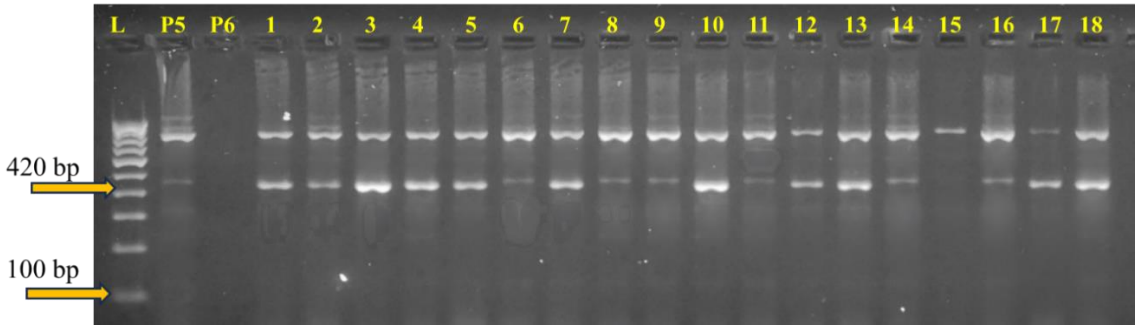

Plate 5. PCR amplification pattern observed in KDS 1201 (P_5) × TGX 709-50E (P_6), in 18 F_2 plants using gene specific *titi* primer, L- StepUp 100 bp DNA ladder

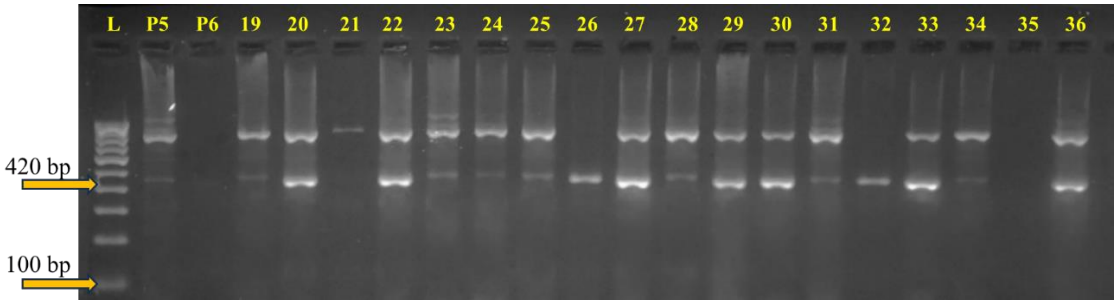

Plate 6. PCR amplification pattern observed in KDS 1201 (P_5) × TGX 709-50E (P_6), in 18 F_3 plants using gene specific *titi* primer, L-StepUp 100 bp DNA ladder

3.2 Molecular Markers-Based Analysis of *lipoxygenase-2* (*lox-2*) Gene in Soybean

In the current study *LOX 2* gene was studied in segregants from (MACS 1520 × NRC 142 and KDS 1201 × NRC 153, crosses using *lox2* gene specific and *Satt 656* primers.

In this investigation, lipoxygenase-free soybean cultivars generated amplicons of various sizes when different primer combinations were utilised.

When the *lx2* specific primer was used, lipoxygenase-free parents showed amplicon fragment of 600 bp in size. The *lx2 specific* primer used in this study, yielded no amplicon in lipoxygenase 2 expressing parents, *i.e.* MACS 1520 and KDS 1201, while an amplicon fragment of 600 bp size was observed in lipoxygenase free parent, *i.e.* NRC 142, NRC153.

When the *Satt 656* primer was used, amplicon of 170 bp (*Lox2Lox2*) size was observed in lipoxygenase 2 expressing parent (MACS 1520), an amplicon of 173 bp (*lox2lox2*) size was observed in lipoxygenase 2 free parent (NRC 142). In KDS 1201 amplified 150 bp allele (*Lox2Lox2*), and a null allele linked 156 bp (*lox2lox2*) *i.e.* NRC 153. Presence of both the alleles in any segregant indicates heterozygosity *Lox2lox2.*

3.2.1 Molecular studies with *lx2* specific primer

3.2.1.1 MACS 1520 × NRC 142

In the cross having MACS 1520 as female and NRC 142 as male parent, NRC 142 which is free from lipoxygenase 2 yielded 600 bp amplicon. The molecular study of 48 plants in which 21 plants are of F_1 generation and 27 plants are of F_3 generation.

The molecular assay of 21 F_1 plants showed that only six F_1 plants *viz*., #1, #4, #5, #9, #11 and #17 exhibit the 600 bp amplicon indicating free from *Lox-2* gene (Plate 7).

The same cross 27 F_3 progenies evaluation by *lx2* specific primer revealed that only 12 F_3 progenies (#22, #23, #30, #31, #32, #37, #38, #39, #40, #41, #42 and #47) which yielded amplification of 600 bp amplicon, were free from *Lox-2* (Plate 8).

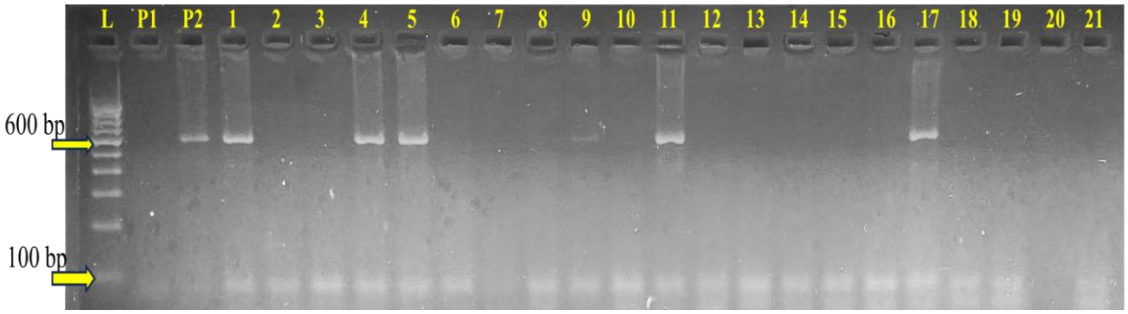

Plate 7. PCR amplification pattern observed in MACS 1520 (P_1) × NRC 142 (P_2), in 21 F_1 plants using *lx2* specific primer, L- StepUp 100 bp DNA ladder

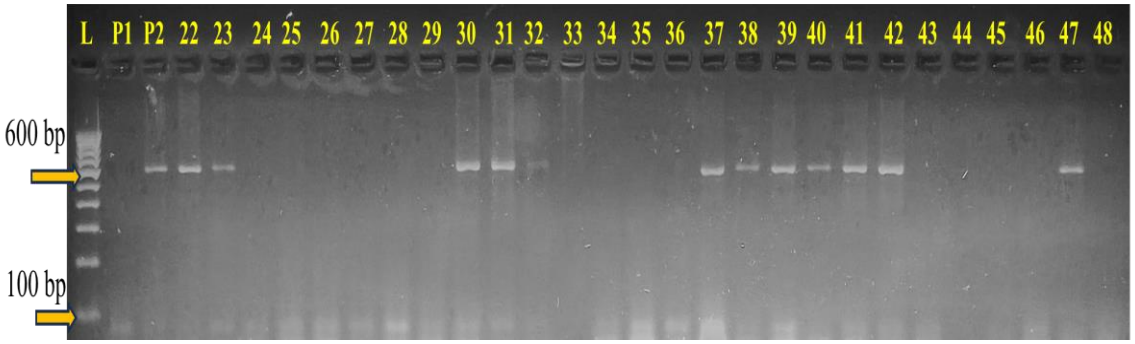

Plate 8. PCR amplification pattern observed in MACS 1520 (P_1) × NRC 142 (P_2), in 27 F_3 plants using *lx2* specific primer, L- StepUp 100 bp DNA ladder

3.2.1.2 KDS 1201 × NRC 153

The second cross having parent female as KDS 1201 and male as NRC 153 which is free for *lox* gene (*lox2* and *lox3*) of both alleles. A total of 27 (13 F_2 and 14 F_3 progenies) segregating progenies of this cross were studied by *lx2* specific molecular marker. In case of 13 F_2 progenies only five progenies #4, #6, #9, #10 and #13 amplified only the 600 bp bands of *lox-2* allele (Plate 9). In case of 14 F_3 plants only three progenies #20, #26 and #27 shows the amplicon (600 bp) of same size as parent NRC 153 (Plate 10). Jin et al*.,* (2012) have reported soybean *lipoxygenase-2* gene variations in terms of molecular sequence. In their investigation, lipoxygenase-free soybean cultivars generated amplicons of various sizes when different primer combinations were utilised. When the *lx2* specific primer was used, lipoxygenase-free parents showed amplicon fragment of 600 bp in size as observed in the present investigation.

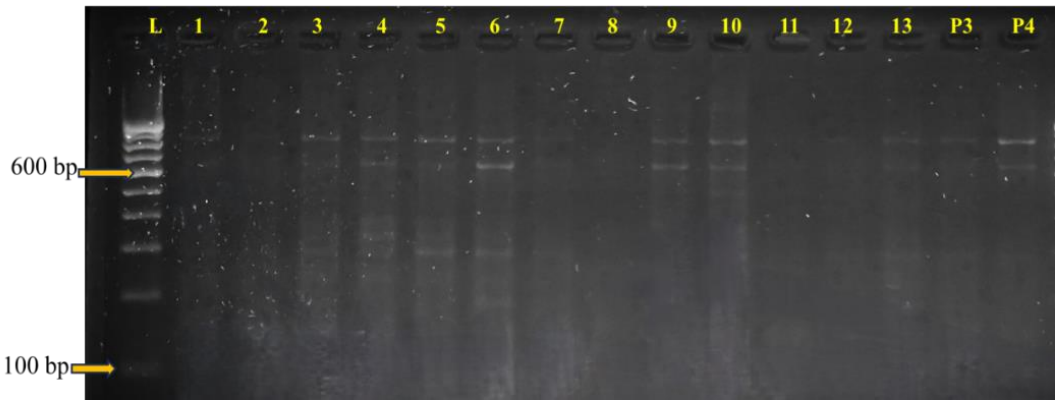

Plate 9. PCR amplification pattern observed in KDS 1201 (P_3) × NRC 153 (P_4), in 13 F_2 using *lx2* specific primer, L- StepUp 100 bp DNA ladder

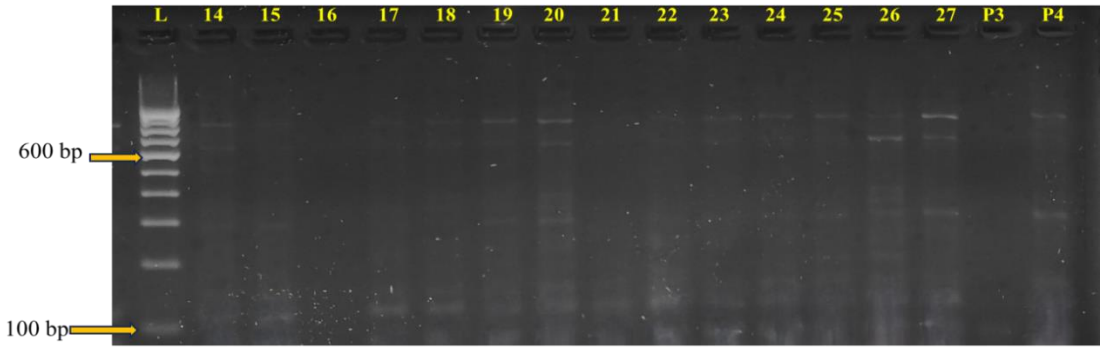

Plate 10. PCR amplification pattern observed in KDS 1201 (P_3) × NRC 153 (P_4), in 14 F_3 using *lx2* specific primer, L- StepUp 100 bp DNA ladder

3.2.2 Molecular studies with *Satt 656* primer

Total of 75 plants derived from crosses MACS 1520 × NRC 142 (48 plants) and KDS 1201 × NRC 153 (27 plants) were studied with *Satt 656.* The primer is co-dominant hence can able to amplify the both dominant and recessive alleles for the null lipoxygenase factor. So in the MACS 1520 × NRC 142, *Satt 656* primer can amplify 170 bp amplicon which showing homozygous dominant allele *Lox2Lox2*, for 173 bp amplicon showing homozygous recessive (null) allele *lox2lox2* and the heterozygous individual exhibit both 170 bp and 173 bp amplicons (*i.e. Lox2lox2)*. In KDS 1201 × NRC 153, parents *Satt 656* amplified one at 150 bp indicating presence of *Lox2* allele (KDS 1201**)** and at 156 bp indicating null *lox2* allele (NRC 153).

3.2.2.1 MACS 1520 × NRC 142

The 48 progenies *i.e.* 21 F_1 and 27 F_3 progenies were analysed by using the *Satt 656* primer. In the context of 13 F_1 progenies (#2, #3, #6, #8, #10, #12, #13, #14, #15, #16, #18, #19 and #21) exhibit 170 bp amplicon with homozygous dominant allele and 6 F_1 progenies (#1, #4, # 5, #9, #11 and #17) exhibit both amplicons as they are heterozygous and no amplification was observed in two progenies (#7 and #20) (Plate 11). The molecular assay of 27 F_3 plants revealed that, 13 progenies (#24, #25, #26, #27, #28, #29, #34, #35, #36, #43, #45, #46, #48) show a 170 bp, one progeny was found heterozygous (#47), 11 progenies (#22, #23, #30, #31, #32, #37, #38, #39, #40, #41, #42) amplified a 173 bp recessive homozygous (*i.e.* null *lox-2* allele) while two progenies (#33, #44) did not showed any amplification (Plate 12). The (Kumar et al*.,* 2021) was also reported similar result for *Satt 656* primer.

3.2.2.2 KDS 1201 × NRC 153

The present cross having NRC 153 parent was used as male which is null for *lipoxygenase* gene (*lox2lox2*). In this cross total of 27 segregating progenies were evaluated for the *lox* gene. Among that 27 progenies the 13 progenies were F_2 and 14 progenies were from F_3 generation. In this parents *Satt 656* amplified amplicon at 150 bp presence of *Lox2* allele (KDS 1201**)** and at 156 bp indicating null *lox2* allele (NRC 153).

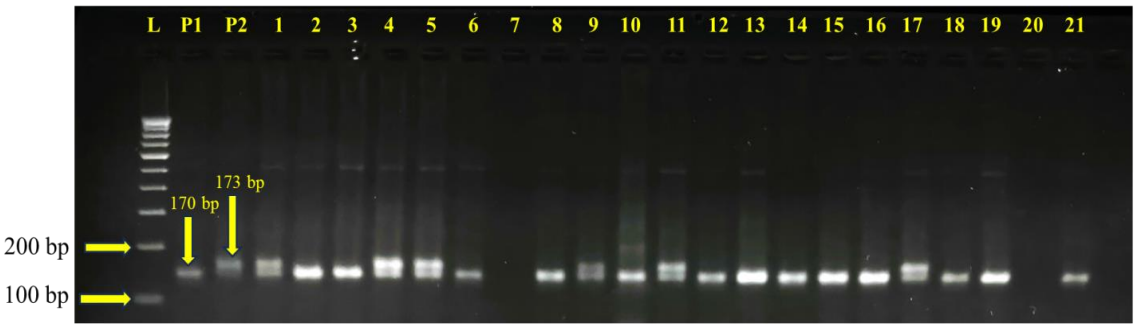

Plate 11. PCR amplification pattern observed in MACS 1520 (P_1) × NRC 142 (P_2), in 21 F_1 plants using *Satt 656* primer, L- StepUp 100 bp DNA ladder

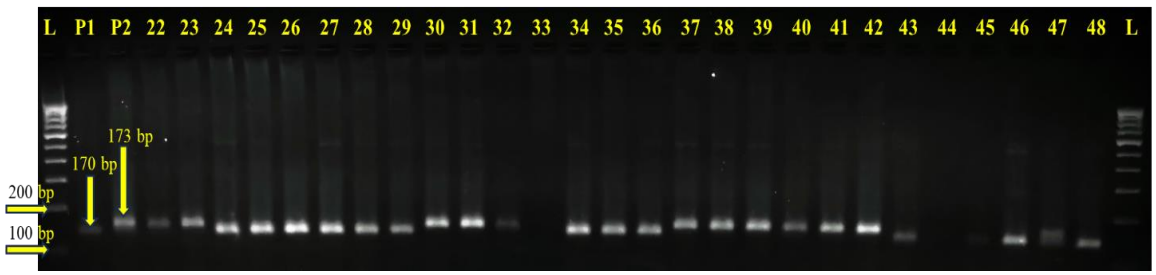

Plate 12. PCR amplification pattern observed in MACS 1520 (P_1) × NRC 142 (P_2), in 27 F_1 plants using *Satt 656* primer, L- StepUp 100 bp DNA ladder

In the context of F_2 plants out of 13 plants assayed, 9 F_2 plants ((#1, #2, #3, #5, #6, #8, #9, #12 and #13) showed the amplicon only at 150 bp as they homozygous dominant which are expressing the *Lox 2* gene, the 3 plants (#4, #7 and #11) were heterozygous for the gene as amplified both amplicon and only one plant (#10) amplified only 156 bp was completely recessive homozygous for null *lox2* allele *i.*e. free from *lox-2* gene which is desirable (Plate 13). In 14 F_3 plants scenario for same cross similar pattern of amplification was observed. In which 9 plants ((#15, #16, #17, #19, #20, #21, #22, #24 and #25) have 150 bp amplicon as they expressing *Lox-2* gene, two plants ((#18 and #27) were heterozygous for the gene as amplified both amplicon, two plants (#23 and #26) amplified 156 bp amplicon only so are the free from Lox-2 (*i.e.* null *lox2* allele) and one plant (#14) did not yielded any amplification (Plate 14).

Rawal et al., (2020) investigated the genetic removal of Lox-2 from 'JS97-52' using marker-assisted introgression of the Lox-2 null allele from PI596540 (*lox2lox2*). Foreground selection in BC_1F_1, BC_2F_1, and BC_3F_1 of the cross 'JS97-52' × PI596540 was carried out using *Satt 656*. In BC_2F_2 and BC_3F_2, homozygous recessive plants (*lox2lox2*) were identified using SSR marker which matches the our findings.

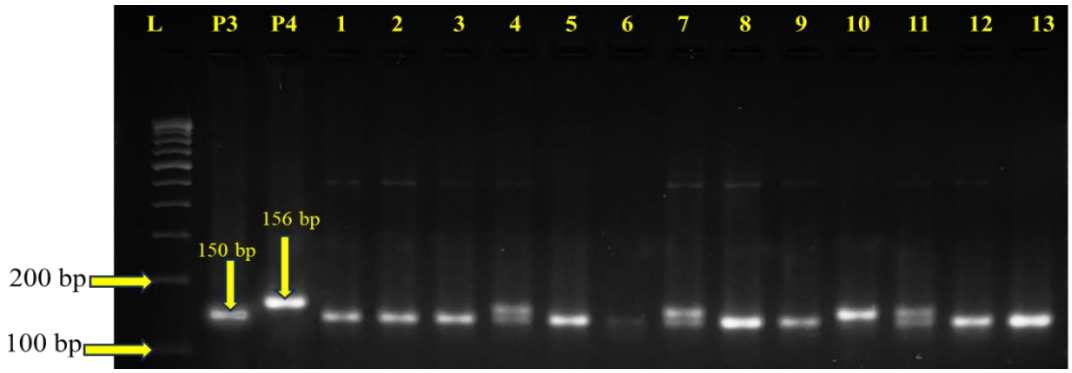

Plate 13. PCR amplification pattern observed in KDS 1201 (P_3) × NRC 153 (P_4), in 13 F_2 plants using *Satt 656* primer, L-StepUp 100 bp DNA ladder

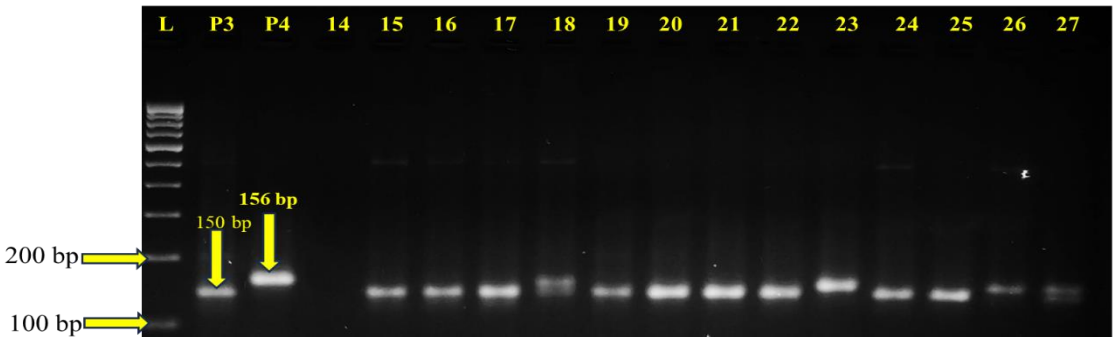

Plate 14. PCR amplification pattern observed in KDS 1201 (P_3) × NRC 153 (P_4), in 14 F_3 plants using *Satt 656* primer, L-StepUp 100 bp DNA ladder

3.3 Molecular Markers Based Analysis of *lipoxygenase-3* (*lox-3*) Gene in Soybean

In this assay the gene specific primer *Lox-3-3′* was used. *Lox-3-3′* gene specific marker distinguished the homozygous dominant (*Lox3Lox3*), homozygous recessive (*lox3lox3*) *i.e.* null allele and heterozygous (*Lox3lox3*) plants by generating PCR fragments of 396 bp, 476 bp and both the amplicons, respectively. The molecular assay was carried out for the cross KDS 1201 × NRC 153 as male parent, NRC 153 is null (*lox3lox3*) for *Lox3* gene indicating free from lipoxygenase-3 activity. Total of 13 F_2 and 14 F_3 progenies of this cross were studied for evaluating the presence/ absence of *lox 3* gene alleles.

Among 13 F_2 plants evaluated, 4 plants (#2, #3, #5 and #9) amplified 396 bp amplicon as they having *Lox3Lox3* genotype and are positive for their activity, 5 plants (#1, #4, #7, #8 and #12) were heterozygous for same gene and 4 plants (#6, #10, #11 and #13) amplified 476 bp amplicon expressing *lox3lox3 i.e.* null alleles, which indicates that they are free from lipoxygenase-3 activity (Plate 15).

Similar results were obtained in 14 F_3 plants as 2 plants ((#19 and #25) amplified at 396 bp, 4 plants ((#18, #20, #24 and #26) were heterozygous with both amplicon and 7 plants (#15, #16, #17, #21, #22, #23 and #27) showed 476 bp as like male parent hence are promising Lox-3 free progenies (Plate 16).

The results of PCR amplicons found to be contrast with previous research of Kumar and Rani., (2019). In their study the *Lox-3-3′* gene specific marker distinguished the homozygous dominant (*Lox3Lox3*) by generating PCR fragments of 476 bp whereas homozygous recessive (*lox3lox3*) generated PCR fragments of 396 bp. The heterozygous (*Lox3lox3*) amplicons showed the presence of both the alleles of 476 bp and 396 bp.

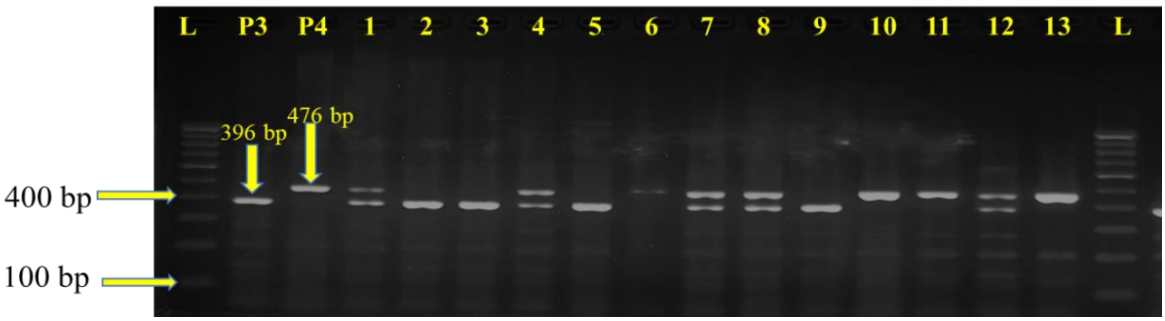

Plate 15. PCR amplification pattern observed in KDS 1201 (P_3) × NRC 153 (P_4), in 13 F_2 plants using *Lox-3-3′* gene specific primer, L- StepUp 100 bp DNA ladder

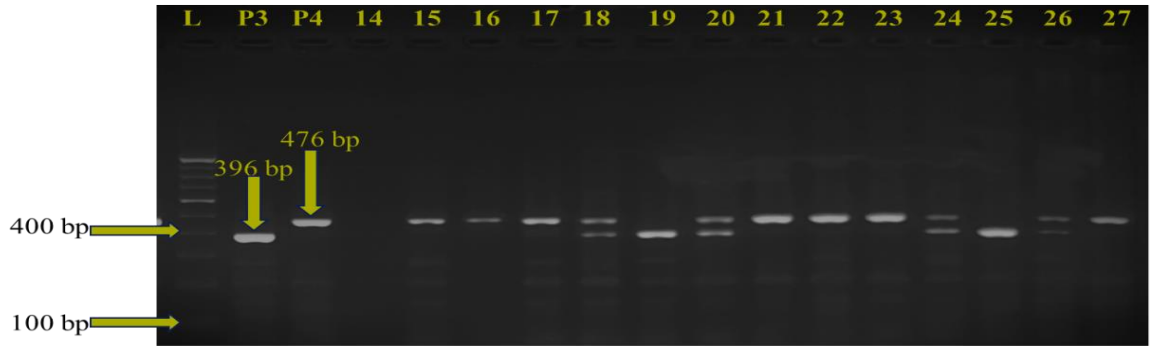

Plate 16. PCR amplification pattern observed in KDS 1201 (P_3) × NRC 153 (P_4), in 14 F_3 plants using *Lox-3-3′* gene specific primer, L- StepUp 100 bp DNA ladder

4. CONCLUSION

The three markers (*titi* gene specific, *lx2* gene specific and *Satt 656*) combined analysis for cross MACS 1520 x NRC 142 revealed that out of 27 F_3 progenies analysed the only three (#30, #32 and #38) were double null (*tititilox2lox2*) indicating free from both the unwanted gene so

can be utilized as a homozygous line for further uniform line development and industrial application.

In KDS 1201 × NRC 153 cross these three-marker analysis indicates that amongst 13 F_2 progenies only single progeny (#10) found to be double null (*tititilox2lox2*), while among 14 F_3 progeny for same cross only two progenies (#23 and #26) were double null (*tititilox2lox2*) that have both *titi* and *lox2lox2* alleles.

In KDS 1201 × NRC 153 four markers (*titi* gene specific, *lx2* gene specific, *Satt 656* and *Lox-3-3'*) combined analysis reveals that two progenies #10 (from F_2 generation) and #23 (from F_3 generation) were found triple null (*tititilox2lox2lox3lox3*) genotype means these two plants were free from KTI, Lox-2 and Lox-3, so are more promising in development of healthy consumable soybean lines in breeding program and also for soy product in the industries.

In KDS 1201 × TGX 709-50E cross only single marker analysis of *titi* specific primer revealed out of 36 plants (both F_2 and F_3 generation) only 18 plants were carry null *KTI* allele so can be in further utilized to convert into double and triple null lines.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declares that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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