

KASP-Assisted pyramiding of Null *KTI* and Null *Lox2* with YMD resistance in Indian soybean

Kamalpreet Singh Mandahal^{1*}, Karmvir Singh Garcha¹, Harmandeep Kaur¹, Asmita Sirari¹, Satinder Kaur¹, Vineet Kumar² and Balwinder Singh Gill¹

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Abstract: *The current study was planned to pyramid Yellow Mosaic Disease (YMD) resistance with null KTI and null Lox2 in soybean, with a purview to create food grade soybean genotypes which can sustain under heavy disease pressure of North Indian plains. Hence, a cross was made between NRCSL 3 (R) x NRC 142 (S, null KTI + null Lox2). F₂ and subsequent F₅, was screened using newly developed functional KASP markers for both traits. The markers were validated on a panel of F₁ hybrids (derived from 3 different crosses) as well as wild type and double null parents. Using the markers, a total of 15 double null lines i.e., null KTI + null Lox2; having YMD resistance were identified. Two of these outyielded the best check and parent NRCSL 3 in terms of economic yield. This is the first report of pyramiding of YMD resistance with null KTI and null Lox2.*

Keywords: Marker assisted pyramiding, null KTI, null Lox2, Yellow mosaic disease (YMD), soybean

INTRODUCTION

Soybean, an oilseed legume, is a highly valuable crop grown throughout the world. It ranks first as an oilseed crop in the world. In terms of production, Brazil overtook USA in 2019-20, and is currently the global front-runner; producing approximately 39% of the world's harvest. Other than Brazil, US, Argentina and China are the leaders of soybean production. India is ranked at fifth position worldwide in soybean production (USDA 2024). World production of soybean during 2023 was 422.3 million metric tons (mt) from an area of 143.3 million hectares and productivity was estimated to be 2.95 ton per hectare (USDA 2024). In India, it occupies about 12.8 million hectares with a production of 12.2 million tonnes (USDA 2024). The Central part of India (lat 21° 6' N; long 26° 30' N) is the hub of soybean production. Under Indian conditions main season of soybean cultivation is from June to October (rainy/kharif season).

Soybean has versatile end uses including its use as human food, animal feed and industrial purposes ranging from cosmetics to tyres. On an average, soybean contains 40% protein, 20% oil and is rich in lysine and vitamins A, B and D. Its oil comprises 85% unsaturated fatty acids (Anderson and Wolf 1995). Apart from the basic nutrients, soybean seeds contain certain nutraceutical components i.e., isoflavones, lecithin, tocopherols, saponins etc., that are known to reduce the risk of certain diseases (atherosclerosis, breast cancer, diabetes, osteoporosis etc.) (Anderson and Wolf 1995). Because of all these



***Corresponding author:**

E-mail: kamalpreet-pbg@pau.edu

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¹ Punjab Agricultural University, Plant Breeding and Genetics, Ferozepur Road, Ludhiana, Punjab 141027, India

² ICAR, National Soybean Research Institute, Khandwa Road, Indore, Madhya Pradesh 452001, India

benefits, soybean has been crowned as Golden bean, yellow jewelry, great treasure beans and miracle beans (Friedman and Brandon 2001).

The use of soybean as food is restricted primarily to China, Taiwan, Japan and Korea. In India, less than 10% of the soybean produced is further processed into soy foods. Presence of antinutritional factors in soybean seed is the key reason behind low acceptance of soy-based foods. The Kunitz trypsin inhibitor (KTI) (20kDa) is the main trypsin inhibitor as well as the primary antinutritional compound (Clemente et al. 2013). These protease inhibitors, depending upon the genotype, comprise around 8-25% of the protein present in seed, and have been associated with adverse effects like inhibition of growth and anomalies in pancreas *viz.*, hyperplasia and hypertrophy in mammals (Liener 1995). The KTI, with about 80% of the trypsin inhibitor activity, is the main culprit of ill effects of soy food and needs to be inactivated before soy food consumption. Along with the KTI and some other components, the off/beany flavour accompanying soy foods is a key factor for repugnance of the bean in daily food chart. This off/beany flavour arises when linoleic and α -linolenic acids are oxidized by the native lipoxygenase enzymes. This reaction produces hydroperoxides which are further acted upon by hydroperoxide lyases, releasing volatile compounds (aldehyde and ketone) responsible for off/beany flavour associated with the soy products (Baysal and Demirdoven 2007). There are three isoforms of seed lipoxygenase enzyme in soybean *i.e.*, lipoxygenase-1, -2 and -3. Amongst the three lipoxygenases enzymes, lipoxygenase-2 (Lx2) has been found to be mainly responsible for n-hexanal (aldehyde) production, which is the primary source of beany flavour (Nishiba et al. 1995). Thus, *Lx2* gene is the major driver of off/beany flavour in soy foods. Heat treatment is a commonly used method of inactivation of these enzymes; however, it is a cost ineffective solution, accounting for substantial part of the total processing costs (Kumar et al. 2015). Also, antinutrient residual activity of some level remains; dependent upon the treatment time and temperature (Friedman and Brandon 2001). More importantly, thermal treatment of 20 minutes which inactivates ~90% of the inhibitor results in 20% decline in protein solubility (Anderson 1992). Genetic elimination of KTI and *Lx2* enzymes will lead to development of food grade varieties with lower energy requirement and thus leading to reduced cost of processing. The genotypes which lack KTI and *Lx2* will certainly attract processors and consumers alike.

DNA based markers have improved the efficiency and accuracy of breeding programmes since their advent. These markers have been used for a long period for transfer of desirable traits by tagging the genomic locations responsible for the phenotype in question. While the task of phenotyping (or biochemical analyses) is lengthy and/or stage specific, DNA based markers are free from these limitations, and thus are preferred whenever available. Gel based markers specific to the null alleles of both the *KTI* (Moraes et al. 2006) as well as *Lx2* (Shin et al. 2012) have been used in some studies. However, these are dominant markers and fail to distinguish between homozygous and heterozygous individuals, which reduces their efficacy particularly for marker assisted breeding. Single nucleotide changes, associated with the trait of interest can be converted into Kompetitive Allelic Specific PCR (KASP) markers. Low cost, high specificity and high throughput have led to KASPs being the marker of choice of the current era (Esuma et al. 2022).

North-West part of India is a potential area for expansion of soybean. Particularly, Punjab state is a latent hotspot for soybean-based cottage industry *viz.*, Tofu and soya-milk production. However, the progress so far has been hindered by lack of suitable high yielding and food grade varieties along with Yellow Mosaic Disease (YMD), a major constraint in soybean production. YMD is transmitted by whitefly (*Bemisia tabaci*) and the disease can cause upto 100 per cent losses depending upon cultivar and the crop stage of incidence (Wrather et al. 1997).

NRC 142 [Pedigree: (JS 97-52 $\times$ PI 596540) $\times$ PI 542044] is a food grade variety which has null alleles for *KTI* and *Lx2*. Also, it has stable yields across multiple environments in India and has been recommended for Central zone and South zone. However, it is susceptible to YMD and therefore is not suitable for North Indian conditions particularly Punjab, where high incidence of YMD is reported. Another variety NRCSL 3 [Pedigree: SL 958 $\times$ NRC 94], was recommended in 2024 for North plain zone of India as a high yielding variety with resistance against YMD. These genotypes were chosen as donors for marker assisted pyramiding of null *KTI* and null *Lx2* along with phenotypic screening for YMD resistance. KASP markers were developed for the marker assisted selection of null genotypes. Segregating populations (F_2 , $F_{2:3}$ and F_3) developed from cross of NRCSL3 $\times$ NRC 142 were used for pyramiding of null *KTI* and null *Lx2* with YMD resistance. Hence, the current study aimed at identification of agronomically superior; YMD resistant; double null genotypes to develop a food grade soybean variety.

MATERIAL AND METHODS

Experimental materials

The current study was executed at Punjab Agricultural University, Ludhiana (Punjab, India). Field and offseason experiments were carried out at experimental field area of Pulses Section, Department of Plant Breeding and Genetics (lat 30° 54' 42.926" N, long 75° 47' 11.663" E). Genotyping experiments were performed in labs of School of Agricultural Biotechnology. The cross between NRCSL 3 (♀, resistant against YMD) and NRC 142 (♂, null *KTI*, null *Lox2*) was made during *kharif* season of 2020. 15 F₁s obtained from the aforementioned cross were grown in offseason nursery during winter 2020-21. F₂ population of 1573 plants, derived from these F₁s was raised in *kharif* 2021, further F_{2:3} population was planted in *kharif* season of 2022 and from this population, 181 single plant selections (SPS) were made based on agronomic desirability. All of these 181 SPS were advanced in an offseason nursery (F₄ generation) during winter 2022-23 and F₅ progenies were planted in *kharif* 2023. These progenies were labeled as per the respective F₂ number, with "20-3" (Year of the cross - cross number) as the prefix. Besides the aforementioned cross, a panel of F₁s derived from NRCSL 3 × NRC 142 (8 F₁s), SL 955 × NRC 142 (10 F₁s) and PS 1347 × NRC 142 (10 F₁s) was used to validate the KASP markers developed in the current study. Two non-null (wild type for *KTI* and *Lox2*) genotypes PS 1670 and SL 958, and the parents of the above crosses were also included in the panel as controls.

DNA extraction, quantification and dilution

DNA was extracted from the 1573 F₂ plants, validation panel and 173 F₅ progenies (as a composite sample from 5 tagged plants) using the CTAB method. Agilent Biotek Take3 spectrophotometer was used to determine the DNA quantity. After giving RNase treatment, agarose gel-based quantification was performed to ensure that RNA was eliminated. The working solutions of DNA were prepared having final concentration of 35-50 ng/μl in nuclease free water.

Development of KASP markers for *KTI* and *Lox2*

KASP markers were developed for both the genes by using prior reports for determining SNP sites. The mutation in the *KTI* gene leading to null *KTI* (*kti*) was described by Jofuku et al. in 1989. The SNP site has been validated via conventional primers by Moraes et al. (2006). In the *KTI* gene (Glyma.08g341500), G has been converted into T at base position 481 and was used to design the primers given in Table 1. Due to a deletion at bases 486 and 487,

Table 1. Sequences of forward primers and common primers for *KTI* and *Lox2*

Sr. No.	Gene	Primer
		Sequence
1	KTI	KKKtiF1
		GAAGGTCGGAGTCAACGGATT CAATGGATGGTTGGTTTAGACTTG
KKKtiF2		
GAAGGTGACCAAGTTCATGCT CAATGGATGGTTGGTTTAGACTTT		
KKKtiCP		
3		ACATTGTGCATCCTCAGCTTGC
4	Lox2	KKLox2-1-CP
TCCATCTATGATGTATGTTATGTCTCA		
KKLox2-1-R1		
GAAGGTCGGAGTCAACGGATT TGAATGGCTCAATCACC GCA		
KKLox2-1-R2		
GAAGGTGACCAAGTTCATGCT TGAATGGCTCAATCACC GCT		
KKLox2-2-F1		
GAAGGTCGGAGTCAACGGATT TTATTTTGTT CATAGGTTAAATACTCAT		
KKLox2-2-F2		
GAAGGTGACCAAGTTCATGCT TTATTTTGTT CATAGGTTAAATACTCAA		
9		KKLox2-2-CP
		TTATAAATTGGGTGAAGAGCACTAA

primers were designed only in the forward direction. Primer KKKtiF2 amplifies the null allele, while KKKtiF1 anneals with the wild type.

In the null *Lox2* allele (Glyma.13g347500), the protein sequence differs from the wild-type in the substitution of glutamine (Gln) for the histidine (His) residue at position 532. These results were validated by Reinprecht et al. (2011) and Shin et al. (2012) in different materials. Hence the SNP site (T→A) was used to design primers in both orientations i.e., both forward and reverse primer sets were designed (Table 1). Primers KKLox2-1-R2 and KKLox2-2-F2 were designed to amplify the null allele. BLASTn against soybean genome was used to confirm *in silico* specificity of the markers. The primers were designed using Primer3 (Untergasser et al. 2012) and marker synthesis was outsourced from BarcodeBioSciences.

KASP genotyping for *KTI* and *Lox2*

KASP genotyping was carried out using a Veriti 384-Well Thermal Cycler (Applied Biosystems) in a reaction volume of 4 μ L. Each reaction comprised 2 μ L of genomic DNA at a concentration of 50 ng μ L⁻¹, 1.946 μ L of 2 $\times$ low ROX KASP master mix (LGC, Middlesex, UK), and 0.056 μ L of primer mix. The PCR protocol included initial denaturation step at 95 °C for 15 minutes, followed by touchdown cycling: 10 cycles of 95 °C for 20 seconds, 66 °C (with a 0.6 °C decrease per cycle) for 25 seconds, and 72 °C for 15 seconds. This was followed by 35 additional cycles of 95 °C for 10 seconds, 60 °C for 1 minute, and 72 °C for 15 seconds, with a final extension at 72 °C for 5 minutes. After amplification, the plates were read using an Infinite M200Pro plate reader (Tecan Group Ltd.), and allele calls were assigned using KlusterCaller software (LGC, Middlesex, UK).

Agronomic traits and disease reaction of progenies

The 181 F₅ progenies were sown in an augmented design during *kharif* 2023 along with 5 check genotypes viz., SL 958, SL 955, SL 1074, NRCSL 3 and SL 979. Five random plants taken from each progeny were tagged for recording agronomic data on days to flowering, days to maturity, plant height (cm), biological yield (g plant⁻¹), economic yield (g plant⁻¹), harvest index (%), and hundred seed weight (g). PAU, Ludhiana is the hot spot for YMD incidence. Infector rows using susceptible check JS 335 were planted at regular intervals to ensure sufficient disease pressure. The YMD reaction of progenies was recorded during the first fortnight of August, on per plot basis. Briefly, per cent disease incidence (PDI) was calculated taking percentage of diseased/total plants; severity grade (SG) and corresponding response values (RS) were given based on cumulative disease scores of the plot. Coefficient of infection was calculated as product of PDI $\times$ RS and was used thereon to assign disease reaction (DR) to the genotypes, as per the scale given by Singh and Singh (2000).

RESULTS AND DISCUSSION

A cross was made between NRCSL 3 $\times$ NRC 142 to pyramid the desirable attributes from both the genotypes. Choice of NRC 142 as pollinator was made due to its susceptible nature and the problems in seed production associated with it. The F₂ population grown during *kharif* 2021 was used to plant F_{2:3} rows during *kharif* 2022. Genetic removal of *KTI* and *Lox2* is futile, unless the genotype has improved fitness and good yield. Hence, the F_{2:3} was evaluated for agronomic desirability and out of the large population 181 plants were selected as Single Plant Selections. F₄ population comprising of these 181 SPS were grown in an offseason nursery. The F₅ was grown under field conditions during *kharif* 2023 in an augmented design to evaluate the performance of the SPS vis-à-vis checks and parents. DNA of those F₂ plants, which were predecessors of SPS in F_{2:3} was used for genotyping for null alleles of *KTI* and *Lox2*. A number of F₂ plants were heterozygous for these genes, hence, SPS were advanced from F_{2:3} to F₅ via selfing, to benefit from increased homozygosity owing to additional rounds of selfing. These were then screened genotypically and phenotypically in F₅ to identify pyramided lines having the null alleles of *KTI* and *Lox2* along with YMD resistance.

Genotyping of parents using KASPs

The designed KASP markers were first amplified using parental genotypes in replication and Non-Template Control (NTC). As evident from the KASP assay, markers KKKti and KKLox2-2 were able to distinguish between parental genotypes very efficiently and nice clusters at different axes were observed. The plot of fluorescence reads for KKLox2-1 revealed spurious amplification as both the genotypes were intermingled in the plot; thus, rendering the marker non-informative and useless.

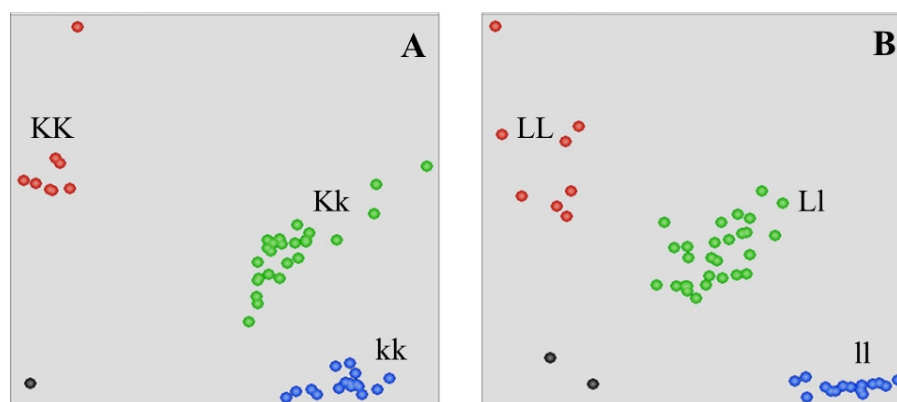


Figure 1. KASP genotyping of the validation panel. Blue and red dots represent homozygous plants, green dots represent heterozygous plants and black dots represent NTC. KK: homozygous for wild type *KTI*, Kk: heterozygous for *KTI*, kk: homozygous for null *KTI*, LL: homozygous for wild type *Lox2*, Ll: heterozygous for *Lox2*, ll: homozygous for null *Lox2*. A: Amplification of KKKti; B: Amplification of KKLox2-2.

Genotyping of experimental material using KASP markers

Validation of the newly developed markers was done using the validation panel of 51 genotypes. The markers were able to distinguish between homozygous parents (null and non-null) and heterozygous hybrids (Figure 1). The two non-null controls, SL 958 and PS 1670, have also been distinguished clearly and plotted along non-null parents NRC SL 3, SL 955 and PS 1347, as red dots. Blue dots represent the double null parent NRC 142, while green dots represent the heterozygous F_1 hybrids from the three different crosses. The validation clearly defined that the markers were fit to genotype F_2 and F_5 populations.

After validation, the markers were used to screen DNA of 181 F_2 plants, which had been selected as SPS from plant to progeny rows in $F_{2:3}$. The KASP assay on F_2 and F_5 have been depicted pictorially in Figure 2. Out of 181 F_2 individuals, for *KTI*, data of 157 could be generated as 24 plants did not amplify any allele. Out of 157 remaining, 35 were null homozygous for *KTI* while 27 were wildtype homozygous and 95 were heterozygous. Similarly, data for *Lox2* gene was generated for 169 plants only; 51 were homozygous for null *Lox2*, while 35 were homozygous wild type, and 83 were heterozygotes. Considering the data of both genes together, 9 F_2 plants were homozygous double null; 43 were double heterozygous; and 8 were double homozygous for the wild type allele.

The population was advanced (by selfing) to select homozygous for null alleles for both the genes in F_5 generation. Eight genotypes confirmed to be wild type for both the alleles in F_2 were removed from genotyping step for F_5 population. After genotyping of 173 F_5 progenies, 48 were *ktikti*, while 66 were *lox2lox2*. 49 and 44 genotypes were heterozygous for *KTI* and *Lox2*, respectively. A total of 19 F_5 progenies were found out to be homozygous double null, i.e., null alleles for both *Lox2* and *KTI* were present.

Disease reaction of the double null progenies

The disease reaction of double null progenies was assessed from the data of F_5 population grown in *kharif* 2023. Eight of these 19 progenies were highly resistant against YMD, while four were in the resistant category. Three progenies were moderately resistant, while four were highly susceptible against the disease. Hence, a total of 15 lines were identified which had YMD resistance pyramided with null *KTI* and null *Lox2*. The 8 highly resistant progenies can be used as genetic stocks to pyramid these 3 genes, viz., YMD resistance gene, null *KTI* and null *Lox2* with other desirable attributes and yield traits and have potential to be released as varieties.

Agronomic performance of double null YMD resistant progenies

To compare the agronomic performance, all the 181 F_5 progenies were grown in augmented design along with parents and five checks. The double null and resistant (DNR) progenies were compared with check genotypes for yield and other

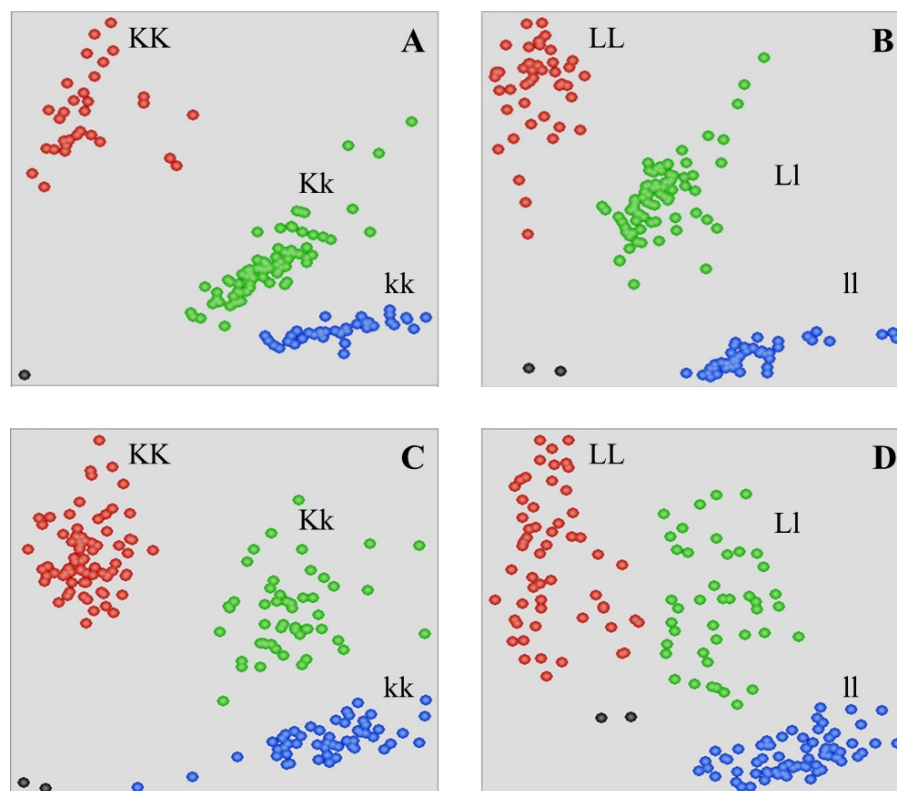


Figure 2. KASP assay using *KTl* and *Lox2* markers. Blue and red dots represent homozygous plants, green dots represent heterozygous plants and black dots represent NTC. KK: homozygous for wild type *KTl*, Kk: heterozygous for *KTl*, kk: homozygous for null *KTl*, LL: homozygous for wild type *Lox2*, Ll: heterozygous for *Lox2*, ll: homozygous for null *Lox2*. A: Genotyping of F_2 using KKKti, B: Genotyping of F_2 using KKLox2-2, C: Genotyping of F_3 using KKKti, D: Genotyping of F_3 using KKLox2-2.

agronomic traits. The adjusted means of the DNR progenies and checks along with least significant interval (LSI) have been given in Table 2. A perusal of the Table 2, reveals two things; first, the DNR progenies exhibited ample variation for all the traits and second, DNR progenies out-performing the best check were identified for different characters. Twelve out of the 15 DNR progenies were significantly different than best check SL 958 for days to flowering; Progenies 20-3-755 and 20-3-1440 were the earliest DNRs to flower (46.52 days). Similarly, for days to maturity, all the 15 progenies were significantly different than the best check; the progeny 20-3-373 was the earliest maturing DNR taking 115.84 days to maturity. In terms of plant height, 3 of the progenies were statistically shorter than the shortest check SL 979 (86.57 cm); while 5 progenies were statistically taller than SL 979. The progenies 20-3-1017 and 20-3-1387 were shortest and tallest amongst the DNRs having respective heights of 71.93 cm and 107.53 cm.

NRCSL 3 was the best check for biological yield per plant, economic yield per plant, harvest index (%) as well as 100-seed weight. Four progenies were statistically superior than NRCSL 3 for biological yield per plant while two progenies were found to be superior for economic yield per plant. The progeny 20-3-761 had highest biological yield (127.39 g) and economic yield (47.47 g) per plant. The progeny 20-3-755 (42.49 %) was best among the 5 DNRs having significantly higher harvest index than the check. Only a single progeny, 20-3-1374, had significantly higher 100-seed weight (14.99 g) than NRCSL 3. Economic yield is the primary trait of importance for the growers. Hence, an elaborate comparison of DNR progenies with respect to economic yield has been done. Out of the 15 DNR progenies, 12 were at par or better than the best check. The progenies 20-3-644 and 20-3-761 statistically out-did NRCSL 3 for economic yield per plant.

The *KTl* and *Lox2* are the main hindrances in popularisation of the soybean as human food. They are directly responsible for increased costs of processing. A double null genotype will be appealing to processors as well as households which

Table 2. Disease reaction and agronomic performance of double null progenies

Sr. No.	Treatment	DR	DF	DM	PH	BY	EY	HI	HSW
1	20-3-334	R	55.12*	130.64*	87.73	79.52	27.33	34.55	12.23
2	20-3-373	HR	47.92*	115.84*	92.52	126.65*	28.17	21.52	9.83
3	20-3-397	MR	50.52*	130.24*	90.81	60.59	24.97	39.30*	12.19
4	20-3-471	HR	63.52*	129.24*	101.61*	67.39	27.17	38.95*	11.56
5	20-3-614	R	58.52	132.44*	94.41	73.96	29.37	39.48*	12.20
6	20-3-644	R	61.92	139.84*	97.52	108.50*	42.07*	38.64*	10.21
7	20-3-755	HR	46.52*	129.24*	79.41	58.59	26.37	42.49*	9.60
8	20-3-761	R	51.52*	122.24*	104.01*	127.39*	47.37*	37.92	10.34
9	20-3-1014	HR	50.52*	142.44*	101.81*	68.16	22.97	33.67	10.84
10	20-3-1017	MR	50.92*	120.84*	71.93*	45.64	15.68	34.86	11.61
11	20-3-1150	HR	48.12*	120.64*	101.73*	102.92*	21.53	20.61	11.12
12	20-3-1374	HR	60.52	142.44*	91.61	65.16	24.37	37.28	14.99*
13	20-3-1387	HR	51.12*	130.64*	107.53*	88.32	23.33	26.33	11.19
14	20-3-1414	HR	52.52*	124.24*	75.41*	89.99	23.57	27.00	12.19
15	20-3-1440	MR	46.52*	126.44*	75.21*	23.36	6.11	27.60	8.54
16	SL 958 (c)	HR	59.60 [#]	136.40 [#]	101.92	69.00	17.08	24.85	10.44
17	SL 979 (c)	HR	66.80	140.00	86.52 [#]	73.96	21.81	29.73	8.01
18	NRCSL 3 (c)	HR	60.20	140.60	103.96	80.08 [#]	26.45 [#]	33.08 [#]	11.51 [#]
LSI			3.28	3.05	20.94	10.01	3.86	4.87	1.58

Abbreviations/Symbols used: LSI - Least Significant Interval, DR - Disease reaction, DF - Days to flowering, DM - Days to maturity, PH - Plant height (cm), BY - Biological yield (g plant⁻¹), EY - Economical yield (g plant⁻¹), HI - Harvest index (%), HSW - 100-seed weight (g). (c) denotes check genotypes, * denotes genotypes statistically better than best check, # denotes best check genotypes. HR - Highly resistant, R - Resistant, MR - Moderately resistant

favour multigrain flours. In India, PI 542044, an accession imported from US, has been widely used as source of the null *KTI* allele. MAS has been used to transfer the null allele into DS 9712 (Talukdar et al. 2014), JS 97-52 (Kumar et al. 2015), DS 9814 (Maranna et al. 2016), SL 525 (Dhaliwal et al. 2021) and Phule Agrani, P. Kimya, and P. Sangam (Pawale et al. 2021). The converted genotypes are either susceptible to YMD or their agronomic performance is not satisfactory. Additionally, two null *KTI* genotypes NRC 101 and NRC 102 have been developed but are susceptible to YMD. Similarly, US accession PI 086023 has been used to introgress null *Lox2* allele into cultivar Samrat (Kumar et al. 2013); while, JS 97-52 has been converted to null *Lox2* by transferring null allele from PI 596540 (Kumar et al. 2021). Genotypes NRC 109, NRC 110 and NRC 132 have been developed as null *Lox2* lines, but these too are susceptible against YMD. A double null genotype, NRC 142, has also been developed by Indian Institute of Soybean Research, Indore; but is highly susceptible to YMD as is evident from data generated in the current study. Importance of YMD resistance cannot be over emphasized in the northern plain zone, since diseased plants are severely hindered from achieving their genetic potential. Thus, YMD resistance is pivotal for a secure crop under North-west India.

Marker assisted selection in conjunction with phenotypic selection was used to pyramid disease resistance with these two desirable null alleles. The earlier reported and utilized markers are gel based SSRs and allele specific dominant markers. SSR markers are highly recognized and were the markers of choice for a long period (Zhong et al. 2021). However, their use is limited across populations when the parents do not exhibit polymorphism for the marker under use. Moreover, being flanking markers, some chances of recombinations and false positives remain, particularly in large populations such as ours. Allele specific markers earlier reported, though are based on effective mutation sites, are dominant markers and thus repeated or supplemented (via SSRs) genotyping is required.

KASP are the marker system of new era and offer many advantages over earlier marker systems such as cost efficiency, simplicity, gel-free etc (Esuma et al. 2022). As for our study, KASPs combined the pros of allele specific markers with SSRs, since these were codominant and based on the active site of mutation leaving no room for error. We have successfully created co-dominant KASP markers for that effect and a total of 15 YMD resistant, double null (null *KTI* and null *Lox2*) F_5 progenies have been identified using the KASP markers from a cross of NRCSL 3 $\times$ NRC 142. These progenies will serve as an important breeding and genetic stock to transfer the 3 traits viz., null *KTI*, null *Lox2* and YMD resistance. KASP

markers for *KTI* gene have been developed and validated by Rosso et al. (2021) in using a different donor, while this is the first report for developing KASPs for *Lox2* gene. This is also the first report of development of pyramided genotypes for these three traits.

CONCLUSION

We have successfully created co-dominant KASP markers for *KTI* and *Lox2* genes. These markers were utilized for pyramiding YMD resistance with null *KTI* and null *Lox2*. 15 progenies were identified which had YMD resistance pyramided with null *KTI* and null *Lox2*. Two of these outyielded the best check and parent NRCSL 3 in terms of economic yield. This is the first report for genotypes having YMD resistance, pyramided with null *KTI* and null *Lox2*. Also, this is the first report of functional KASP markers for *Lox2*, and *KTI* in the respective donors.

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DATASET AVAILABILITY

The datasets generated and/or analyzed during the current research are available from the corresponding author upon reasonable request.

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