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Combining the null Kunitz trypsin inhibitor and yellow mosaic disease resistance in soybean (*Glycine max* (L.) Merrill)

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Abstract: For the wide adoption of soybean varieties, it is desirable, that they are Kunitz trypsin inhibitor (Kti) free and resistant to yellow mosaic disease (YMD). The soybean variety SL525 with YMD resistance was crossed with the YMD susceptible variety NRC101 with a *null kti* allele. The F_5 progeny derived from the cross was screened with two simple sequence repeat (SSR) markers (satt409 and satt322) linked with the *null kti* allele and the YMD resistance, respectively, and one *null kti* allele-specific marker. The presence of both desirable traits was further confirmed with the phenotypic data which showed good correlation with the genotypic data. The yield potential of fourteen such identified genotypes having both desirable traits was either at par or superior to SL525, hence, represent improved versions of SL 525.

Keywords: marker assisted selection; *null kti* allele; SSR markers; YMD

The domestication of crop plants over the years has led to the removal of anti-nutrients to suit the digestive tract of humans. However, the soybean (*Glycine max* (L.) Merrill), also known as miracle bean owing to presence of 40 per cent protein and 20 per cent oil, still harbours many anti-nutrients, of which the most important is the Kunitz trypsin inhibitor (Kti). Kti is known to cause the inactivation of the digestive protease, *i.e.,* trypsin which is essential for the digestion of food (Kunitz 1945). The natural mutation of the *Kti* gene is called the *null kti* which is free from anti-nutrients (Orf & Hymowitz 1979). The *Kti* locus responsible for the anti-nutrition in the soybean was mapped on the A2 linkage group (Kiang 1987; Cregan et al. 1999; Kim et al. 2006). The gene specific and simple sequence repeat (SSR) markers (Satt409 and Satt228) linked with the *Kti* locus have been identified (Kim et al. 2006; Moraes et al. 2006). The SSR markers linked with the *Kti* locus, *i.e.,* Satt409 and Satt228, were further validated by Rani et al. (2011) and, thus, could be utilised for the marker assisted selection (MAS) of the *null kti*. The *null kti* was introgressed into the Indian germplasm, and two genotypes NRC101 and NRC102 carrying the *null* allele of the *Kti* gene have been developed (Rani et al. 2010). These genotypes were an excellent source of the *null kti* allele for breeding soybeans free from anti-nutritional factors. However, both of the genotypes were susceptible to yellow mosaic disease (YMD). In order to utilise these two genotypes in soybean breeding programmes, it was imperative to introgress the *null kti* allele from these genotypes in the YMD resistant background.

YMD in leguminous crops is mainly caused by Legumoviruses. YMD belongs to the family Geminiviridae which is the second largest family of plant

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viruses causing devastating epidemics and to the genus begomoviruses which is largest genera of the family Geminiviridae. YMD is believed to be caused by the four major species of begomoviruses namely, *Mungbean yellow mosaic India virus*, *Mungbean yellow mosaic virus*, *Horsegram yellow mosaic virus* and *Dolichos yellow mosaic virus* (Qazi et al. 2007). These four viruses of leguminous crops are collectively named as Legume Yellow Mosaic Viruses (LYMVs), although, at present, there are a total of eleven species in the LYMV group transmitted by an insect vector, *i.e.*, whitefly *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) (Nene 1973). The viral DNA isolated from soybeans in the central and northern regions of India had an 89% identity with the *Mungbean yellow mosaic India virus* (Usharani et al. 2004). The viral DNA is bipartite (DNA A and DNA B), single stranded and circular (Lazarowitz & Shepherd 1992). YMD transmitted by whitefly is a major constraint in the production and popularisation of soybeans in the northern region of India, causing up to 75% yield losses in soybeans (Sharma et al. 2014). Now, it is alarmingly spreading towards the hub of the soybean production, *i.e.*, Central India. The chemical control of the insect vector is not effective or eco-friendly, therefore, the deployment of host resistance is a viable alternative to managing the disease. Similarly, YMD resistance has also been mapped on the C2 linkage group flanked by two SSR markers, Satt322 and Sat_076 (Khosla 2018). These markers have been validated in YMD susceptible and resistant soybean genotypes and are now available for MAS. In the present study, a cross was made between SL 525 (YMD resistant) and NRC 101 (*null kti* allele) and advanced progenies of this cross were selected for both traits with MAS.

MATERIAL AND METHODS

Plant material. SL525, a YMD resistant variety carrying the *Kti* gene, was crossed with NRC101, a YMD susceptible line carrying the *null kti* allele (Figure 1). The progeny was advanced to the F_4 generation without any selection and a total of 45 F_4 lines were sown in a glasshouse. Selection for the YMD resistance was undertaken through MAS and advanced to the F_5 generation. The F_5 progenies were sown in a randomised complete block design with three replications having a row length of 2 meters, a single row per entry with 45 cm spacing between the rows and a plant to plant distance of 5 cm. These progenies were phenotyped for the YMD resistance in the field and simultaneously subjected to a molecular analysis for the YMD resistance and the *null kti* allele. The seeds from these progenies were analysed for the trypsin inhibitor activity.

MAS for YMD resistance. The genomic DNA of the parents, F_4 and F_5 plants was extracted from young leaves by the cetyltrimethylammonium chloride (CTAB) method (Doyle & Doyle 1990). Polymerase chain reaction (PCR) was carried out using the YMD resistance linked markers Satt322, BARCSOYSSR_02_0423 and BARCSOYSSR_02_0425 (Rani et al. 2017; Khosla 2018) in a thermocycler (Mastercycler® Pro, Eppendorf, Germany). The PCR reaction mix (20 µL volume) consisted of 2.0 µL of DNA (25 ng per µL), 4 µL of a PCR buffer (10×), 1.2 µL of magnesium chloride (25 mM), 3 µL of dNTPs (25 mM), 1.5 µL of a forward primer and reverse primer (30 ng per µL), 0.2 µL of Taq DNA polymerase (5 units/µL) and 6.0 µL of nuclease free water. The PCR profile for the amplification of the genomic DNA by the SSR primer included an initial denaturation at 94 °C for

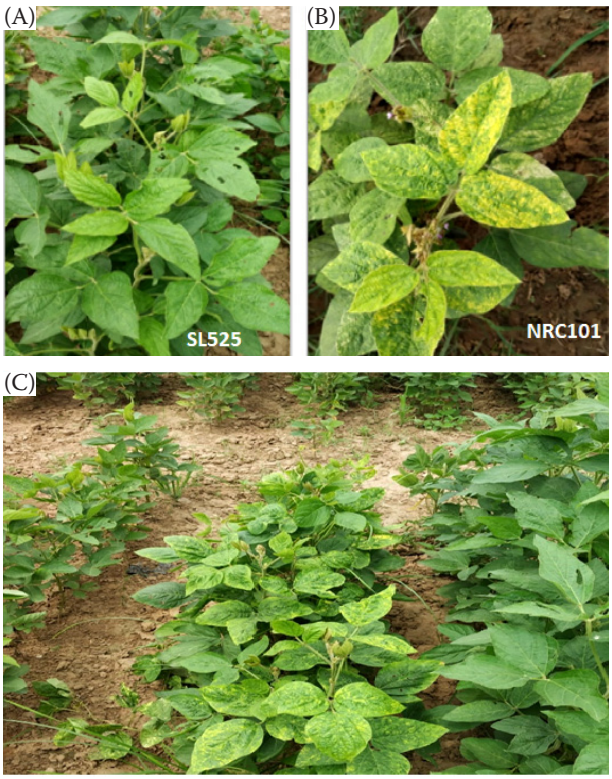

Figure 1. Performance of soybean parents and progenies differing in the yellow mosaic disease (YMD) resistance: SL 525 (resistant parent) (A); NRC 101(susceptible parent) (B); F_3 generation of the cross (C)

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1 min for one cycle, a denaturation at 94 °C for 1 min, annealing for 1 min, elongation at 72 °C for 1 min repeated for 35 cycles and a final extension at 72 °C for 7 min followed by holding it at 4 °C. The primer sequences are given in Table S1 in the Electronic Supplementary Material (ESM). The PCR fragments were separated by agarose gel electrophoresis on a 2.5 % agarose gel and visualised in a gel documentation system (Alpha Imager, USA).

MAS for *null kti*. The genomic DNA of the parents and progenies was amplified with the *null kti* allele specific marker as well as the SSR marker Satt409, linked to the *null kti* (Kim et al. 2006; Moraes et al. 2006). For the *null kti* allele specific primer, the PCR reaction mix (20 µL) comprised of 2.5 µL of the DNA (25 ng/µL), 4 µL of the PCR buffer (10×), 1.2 µL of magnesium chloride (25 mM), 3 µL of dNTPs (25 mM), 2.4 µL of the forward primer and reverse primer (30 ng per µL), 0.2 µL of Taq DNA polymerase (5 units/µL) and 2.9 µL of nuclease free water. For the *null kti* linked SSR marker, the PCR components (20 µL volume) consisted of 2.0 µL of the DNA (25 ng per µL), 4 µL of the PCR buffer (10×), 1.2 µL of magnesium chloride (25 mM), 3 µL of dNTPs (25 mM), 1.5 µL of the forward primer and reverse primer (30 ng/µL), 0.2 µL of Taq DNA polymerase (5 units/µL) and 6.0 µL of nuclease free water. The PCR profile for the amplification of the genomic DNA by the *null kti* allele specific primer included an initial denaturation at 94 °C for 5 min for one cycle, a denaturation at 94 °C for 30 s, annealing 55 °C for 1 min 30 s, elongation at 72 °C for 30 s repeated for 35 cycles and final extension at 72 °C for 7 min followed by holding it at 4 °C. The PCR fragments were separated by agarose gel electrophoresis (1 % agarose gel for the *null kti* allele specific marker and a 2.5 % agarose gel for the SSR marker linked to the *null kti* allele) and visualised in the gel documentation system (Alpha Imager, USA).

Phenotyping for YMD resistance, trypsin inhibitor activity, yield and 100 seed weight. The F_5 progenies were phenotypically screened for the YMD resistance at the hot spot location in Ludhiana, using the infector row technique. A highly susceptible line, *i.e.,* JS 335, was used as the infector row. YMD was scored on 1–9 scale, given by Singh and Bhan (1998) (Table S2 in the ESM). For the Kti phenotyping, the Kti was extracted from soybean seeds using the protocol given by Sadasivam and Manickam (1996) and the trypsin inhibitor activity (TIA) was estimated by the method of Kakade et al. (1974).

For estimating the yield and 100 seed weight, five randomly selected plants from the F_5 progenies in each replication were pooled together and the yield per plant and 100 seed weight were calculated in grams from the mean value.

RESULTS

MAS for YMD resistance. The PCR amplification of both the parents was performed using the YMD resistance linked marker Satt322, BARCSOYSSR_02_0423 and BARCSOYSSR_02_0425. The parents of the cross showed polymorphism with the Satt322 marker, which produced a 200 bp fragment in the resistant parent (SL525) and a 230 bp fragment in the susceptible parent (NRC101). From 45 F_4 progenies, a total of 125 YMD resistant plants were selected based on the co-segregation with the SSR marker (Satt322) (Figure 2). The selected YMD resistant plants were advanced and the F_5 progenies were further validated using the same SSR marker. Out of 125 F_5 progenies, 121 were found to be homozygous for the YMD resistance gene, as indicated by the amplification of the 200 bp fragment, corresponding to a resistant parent. A total of four plants were found to be heterozygous as both the fragments (200 bp and

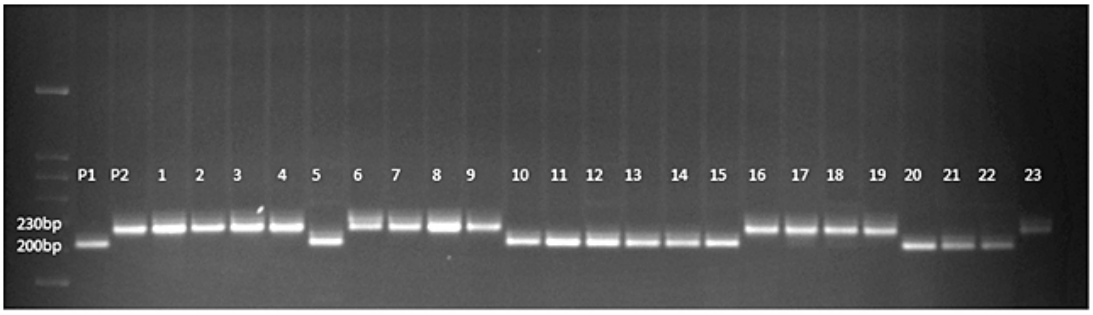

Figure 2. PCR detection with the SSR primer Satt322 linked with the yellow mosaic disease locus in the soybean
P1 – SL525 (200 bp, dominant homozygous); P2 – NRC101 (230 bp, recessive homozygous), and lanes 1 to 23 F_4 population

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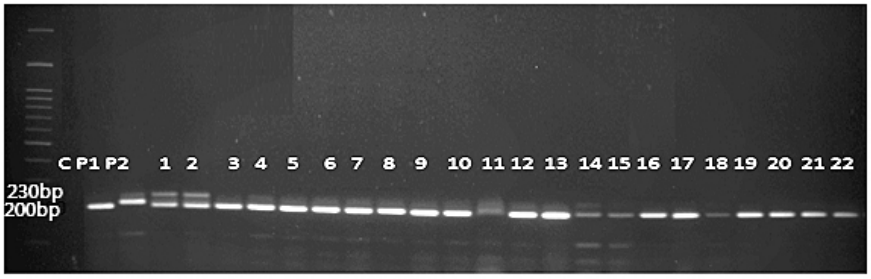

Figure 3. PCR detection with the SSR primer Satt322 linked with the yellow mosaic disease locus in the soybean
C – negative control; P1 – SL525 (200 bp, dominant homozygous); P2 – NRC101(230 bp, recessive homozygous), and lanes 1–22 F_5 plants

230 bp) were amplified (Figure 3). Phenotypically, both the homozygous dominant and heterozygous plants showed a resistant reaction to YMD on a 1–9 scale. The homozygous dominant plants, as well as heterozygous ones, showed a 1 and 3 score on the 1–9 disease scale showing good correlation with the genotypic data (Table S3 in ESM).

MAS for the *null kti* allele. The *null kti* allele specific marker was amplified in 60 genotypes out of 125 F_5 progenies producing a 450 bp fragment. A similar fragment was also observed in the positive control (NRC 101) indicating the presence of the *null* allele (Figure 4). The genotypes showing the presence of the *null* allele could be homozygous recessive as well as heterozygous at the *Kti* locus. Therefore, in order to differentiate the recessive homozygous and heterozygous genotypes at the *Kti* locus, the co-dominant SSR primer Satt 409 linked to the *Kti* gene was used. Satt 409 produced a fragment size of 190 bp in the *Kti* positive parent SL525 corresponding to the dominant homozygous plant and 150 bp in NRC101 (*null kti*) corresponding to the recessive homozygous plant. The primer detected ten genotypes as heterozygous, fifty as homozygous recessive and sixty-five as homozygous dominant (Figure 5). A total of fifty recessive homozygous plants were desirable, whereas the heterozygous and homozygous dominant plants were undesirable. For further conformation, 125 F_5 plants were evaluated with the Kunitz trypsin inhibitor activity. The genotypic data coincided with the phenotypic data, *i.e.*, the trypsin inhibitor activity. The value of the trypsin inhibitor activity was low in the genotypes homozygous for the *null* allele (50 genotypes, 1.4-18.3 TIU/g) and high in the genotypes possessing the functional *Kti* allele (31.2-145.3 TIU/g) in a homozygous state (Table S3 in ESM).

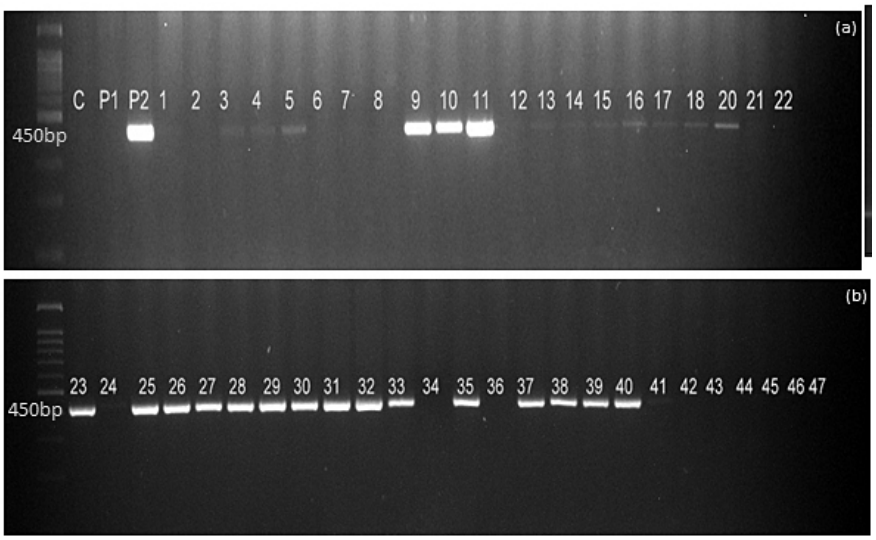

Figure 4. PCR detection with the gene specific primer (*null kti*) in the soybean
C – negative control; P1 – SL525 (no band); P2 – NRC101(450 bp), and lanes 1 to 47 F_5 plants

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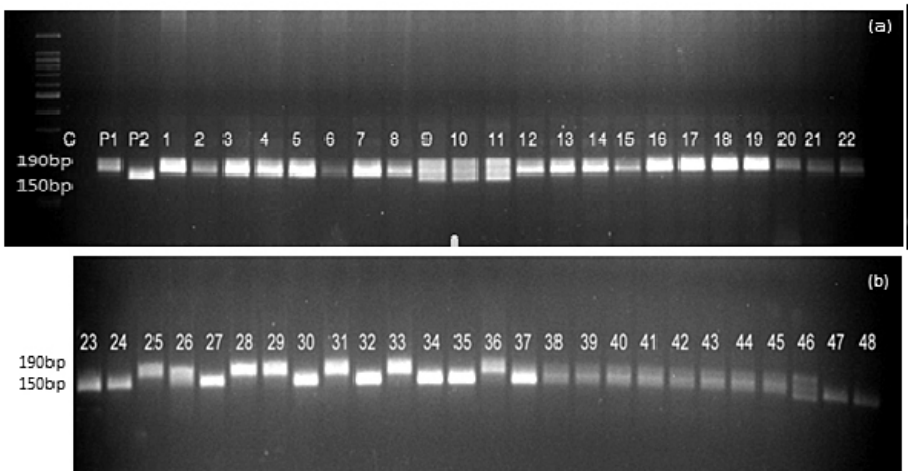

Figure 5. PCR detection with the SSR primer Satt409 linked with the *Kti* locus in the soybean
C – control; P1 – SL525 (190bp, homozygous dominant); P2 – NRC101 (150 bp, homozygous recessive) and 1 to 48 F_5 plants

Selection of promising genotypes. A total of forty-eight F_5 progenies showed homozygosity at the *kti* and YMD resistance locus. In addition to this, they also showed a desirable phenotypic expression for both the traits complementing the molecular markers. Out of these forty-eight progenies, fourteen showed a yield at par with SL525 (Table 1). Therefore, these fourteen genotypes carry both the desirable traits and a good yield potential for further utilisation as donors and represent improved advanced breeding lines.

DISCUSSION

The Kti and YMD are the two main hindrances in popularisation of the soybean. YMD is known to cause severe yield losses and Kti decreases its suitability as

Table 1. Performance of high yielding progenies of soybeans carrying the *null kti* and yellow mosaic disease (YMD) resistance

Genotype No.	TIA (TIU/g)	*Null kti* specific fragment	SSR primer Satt409	YMD score mean of two years	SSR primer Sat322	100 seed weight (g)	Grain yield (g/plant)
SLNR11-03	5.4	P	N	1	R	10.7	37.2
SLNR08-02	9.6	P	N	3	R	8.5	52.3
SLNR13-01	13.5	P	N	3	R	10.7	41.5
SLNR14-04	6.3	P	N	3	R	10.3	35.5
SLNR16-02	5.1	P	N	1	R	11.3	46.2
SLNR16-04	1.6	P	N	1	R	10.1	37.2
SLNR17-01	8.3	P	N	3	R	11.3	43.3
SLNR17-02	3.7	P	N	3	R	8.8	35.3
SLNR18-01	6.4	P	N	1	R	10.4	35.5
SLNR18-03	1.5	P	N	1	R	10.7	40.3
SLNR19-03	6.7	P	N	3	R	11.2	39.9
SLNR20-02	7.3	P	N	1	R	9.9	37.6
SLNR42-02	5.4	P	N	3	R	8.6	37.5
SLNR45-01	9.3	P	N	3	R	10.7	48.7
SL525	97.5	A	K	1	R	8.3	27.3
C.D (5 %)	2.61					1.12	7.77

A – *null kti* absent; P – *null kti* present; N – *null kti* homozygous recessive; K – *Kti* homozygous; H – heterozygous; R – resistance dominant homozygous; S – susceptible recessive homozygous; TIU – trypsin inhibitor units; TIA – trypsin inhibitor activity

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a food purpose. Kti is inactivated in soybeans with a 20 min heat treatment prior to its bio-fortification with wheat flour which is undertaken to enhance the nutritional value of the flour. The development of Kti free genotypes can eliminate the extra cost of heating during the processing and serve as a boon to various processing industries. Furthermore, the rising demand for Kti free soybeans would popularise the soybean crop in India, especially in the northern region of country.

The Kti protein is encoded by the *Kti* gene family and it has ten members present in close proximity, of which three *Kti* genes *viz.*, *Kti*1, *Kti*2 and *Kti*3 are not present in the interceding sequences (Jofuku & Goldberg 1989). *Kti*3 is the main cause expressing the trypsin inhibitor activity in soybean seeds and its maximum expression is observed during embryogenesis while the other two are found in the root and stem as depicted by the mRNA sequences of the same. The sequence of the *Kti*3 gene is 20 % different from the other two genes which are otherwise similar (Jofuku & Goldberg 1989). In the USDA germplasm collection, two soybean accessions (PI157440 and PI196168) were unable to translate the Kti protein (Orf & Hymowitz 1979). A variant of the *Kti*3 gene was present in the accessions, which is a natural mutation, leading to one substitution and two deletions causing a frameshift during the translation of the gene. As a result, there was a premature termination of the *Kti*3 gene and a 100-fold reduction in the production of the Kunitz trypsin inhibitor protein which is an anti-nutritional protein (Jofuku & Goldberg 1989). The variant gene was named as the *null kti* which is a recessive counterpart of the *Kti* gene inherited as a simple Mendelian ratio (Orf & Hymowitz 1979; Hildebrand et al. 1980). In India, the *null kti* allele was introgressed though the MAS approach to develop the nutritionally safe soybean genotypes NRC 101 and NRC 102 (Rani et al. 2010). The major limitation for the utilisation of these genotypes as a *null kti* donor is their susceptibility to YMD.

On the other hand, there are various YMD resistant varieties but none of them are free from the Kunitz trypsin inhibitor. YMD has been mapped in both cultivated and wild genotypes by various workers. Firstly, two SSR markers (Satt301 and GMHSP1790) linked with the YMD resistance were mapped on chromosome 17 (Kumar et al. 2015). Contrary to it, Khosla (2018) mapped two QTLs; one on the D1b linkage group (LG) flanked by BARCSOYSSR_02_0423 and BARCSOYSSR_02_0425 and a second locus on C2 LG flanked by Satt322 and Sat_076. The SSR markers, GMAC7L and Satt322, were reported to be linked to the resistance gene on chromosome 6 by Rani et al. (2017) and the distance between these markers was 3.5 cM (77.115 kb). These markers were further validated by Kumar (2019) and Satt322 was found to be linked with YMD in all the genotypes tested.

The present study was an attempt to combine both traits by crossing a YMD resistant variety, *i.e.,* SL 525, with the *null kti* carrying genotype NRC 101. The fourteen advanced lines from forty-eight F_5 progenies, homozygous for both traits will serve as potential donors for the *null kti* and YMD resistance. Some of these progenies were also promising for the yield and could be developed into commercial varieties.

CONCLUSION

The Kunitz trypsin inhibitor and YMD are the two main constraints in the adoption of soybean varieties on a large scale. The advanced genotypes from the identified F_5 progenies with the *null kti* allele, YMD resistance and high yield have the potential to develop into popular varieties in India.

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