Indian J. Genet., (2021); 81(4): 594-597
doi: 10.31742/ISGPB.81.4.14

ISSN: 0975-6906

www.isgpg.org

SHORT RESEARCH ARTICLE

Incorporation of a null allele of Kunitz trypsin inhibitor through molecular backcross breeding in soybean [*Glycine max* (L.) Merrill.]

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Abstract

Trypsin inhibitors being anti-nutritional, are a major deterrent in the utilization of soybean as a human food and animal feed. For the genetic elimination of Kunitz trypsin inhibitor, a dysfunctional null allele (*titi*) of seed-specific *KTI3* gene was transferred from (NRC 101 and NRC 102) to three rust-resistant varieties, Phule Agrani, P. Sangam, and P. Kimya. Null allele-specific *titi*-420bp and linked Satt409-170bp markers could identify two F_2, single F_3 and F_4 each, eleven BC_1F_2, ten BC_1F_3 and three BC_2F_2 *titi* plants with *titi* genotype among the 131 plants studied. In biochemical assay, the trypsin inhibitor activity in seeds of these plants ranged from 4.03 to 9.67 mg/g^{-1}. Based on both molecular and biochemical studies, it could be concluded that these 28plants were free of Kunitz trypsin inhibitor (*kti* null).

Keywords: Kunitz trypsin inhibitor, Null allele, SSR marker.

Soybean, being protein-rich (40 %), has tremendous potential for alleviating malnutrition among malnourished human population. However, its usage is often restricted due to anti-nutritional trypsin inhibitors that inhibit trypsin activity; with Kunitz trypsin inhibitor contributing for 80% of such activity. Nutritional improvement of qualities of soybean through conventional breeding involves seed destructive biochemical analysis of seed samples (Bernard et al. 1974). Molecular markers provide effective, nondestructive, tissue-independent, rapid analysis of breeding materials. Marker Assisted Selection (MAS) has proved to be efficient for scoring nutritional quality traits that cannot be visually scored. Molecular marker–assisted backcross (MAB) breeding has been employed successfully for transferring few useful quality traits in soybean (Marana et al. 2016; Bernard et al. 2020; Kumar et al. 2020).

The original source of the null *kti* allele is PI 542044 the (Bernard et al. 1991), which is KTI free, as it expresses truncated KTI protein in its seeds. Programs are going on to develop KTI free soybean varieties at the Indian Institute Soybean Research (IISR), Indore (Rani et al.2010; Kumar 1 et al. 2015) and Indian Agricultural Research Institute, New Delhi (Talukdar et al. 2014; Maranna et al. 2016). KTI free trait donors 'NRC101' and 'NRC102' used in the present study were developed at IISR, Indore by Rani 1 et al. (2011), by crossing Kunitz soybean (PI542044) with Samrat. The kti null allele has been successfully introduced through MAS as a monogenic trait.

To utilize Kiti free soybean, the present investigation was undertaken to transfer *titi* null allele in rust-resistant soybean varieties background of P. Agrani, P. Sangam and P. Kimya released by Mahatma Phule Krishi Vidyapeeth (MPKV). The present study comprised of 131 segregating plants (Pawale et al. 2020) derived from crossing three rust-resistant female parents (Phule Agrani, Phule Sangam and Phule Kimya) expressing trypsin inhibitor gene (*TiTi*) with two KTI free male parents (NRC 101 and NRC 102) having its null allele (*titi*), which ICAR-IISR, Indore provided. Leaves of those plants were used for the present investigation to identify KTI free homozygous null (*titi*) and heterozygous (*Titi*) using

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How to cite this article: Pawale S. T., Bhise R. S., Chimote V. P., Deshmukh M. P., Kale A. A. and Naik R. M. 2021. Incorporation of a null allele of Kunitz trypsin inhibitor through molecular backcross breeding in soybean [*Glycine max* (L.) Merrill.]. Indian J. Genet., 81(4):594-597.

Source of support: Nil

Conflict of interest: None.

Received: Dec. 2020 **Revised:** Aug. 2021 **Accepted:** Sept. 2021

molecular markers; and while their seeds were used for biochemical assay (Table 1).

Three primer-pairs, *i.e.,* null allele *titi* specific, SSR Satt 409 and Satt 228, reported to amplify markers linked with KTI free trait (Kim et al. 2006; Kumar 2 et al. 2013; Rani 2 and Kumar, 2015) were used for the study. Null allele-specific *titi* primers amplified a 420 bp band only in KTI free (NRC 101 and NRC 102); while an additional 880 bp band was observed in all parents. Satt-409 primer yielded 170 bp bands in KTI free NRC parents; while 280 bp bands was observed in Phule parents. Satt-228 primer amplified monomorphic 200 bp bands in all of the parents**.** Therefore, only *titi* null allele-specific and *Satt409* markers were used to select KTI free null homozygotes and carrier heterozygotes segregating lines.

Nine of the 39 F2 plants, amplified a *titi* null allele-specific 420 bp band. These included 2 F2 plants from P. Agrani × NRC101; one from P. Agrani × NRC102; two from P. Sangam × NRC101 and four from P. Sangam × NRC102, however on *Satt 409*, amplification was not observed in 5 plants (Fig. 1). It only amplified a 170 bp band in 2 F2 plants derived from P. Agrani × NRC101 and P. Agrani × NRC102; while 31 amplified only a 280 bp band (Fig. 1). Heterozygosity with twin 280bp and 170bp was observed in a F2 plant from P. Agrani × NRC101 and a BC1F1plant of cross P. Kimya ×NRC 102.

Further Satt-409-170 bp marker was observed in two F3 plants (P. Kimya × NRC101) and 4 of the 5 F4 plants from P. Kimya × NRC102, of which 3 F4 plants were heterozygous. Among those amplifying, only 170 bp band included one F3 plant from cross P. Kimya × NRC101 and one F4 from P. Kimya × NRC102 (Fig. 2). In addition, the heterozygosity was observed with both 170bp and 280bp markers in two BC1F3 plants, one each from P. Agrani × NRC 101 and P. Agrani × NRC 102 (Fig. 2). The same plants yielded both *titi*-specific 420bp as well as nonspecific 800 bp bands.

Among the total of 59 BC1F2 plants studied with *titi* gene-specific primer, 54 showed amplification (Fig. 3). Only 18 BC1F2 plants amplified *titi* null allele-specific 420 bp markers including 5 from P. Agrani × NRC101; 7 from P. Agrani × NRC102; 6 from P. Kimya × NRC101 and only one from P. Kimya × NRC102 (Fig. 3). When these59 BC1F2 plants were studied with *Satt409* primer, 48 showed clear amplification (except no amplification in 8 and with faint amplicons in 3 samples) (Fig. 4). Eleven BC1F2 plants amplified only *Satt409-170* bp band while 24 BC1F2 plants amplified only *Satt409*-280 bp band. Among those amplifying only null allele-specific markers, 5 are from cross P. Agrani × NRC101 (#5, #29, #31, #38 and #48), 3 from P. Agrani × NRC102 (#27, #

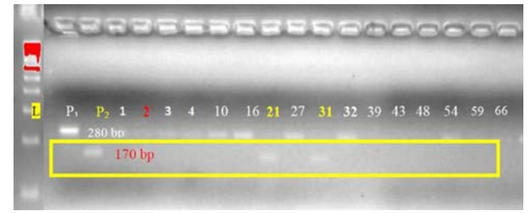

Fig. 1. PCR amplification pattern with Satt 409 primer in F_2 generation, L-Ladder P_1 P. Agrani, P_2 NRC 101;1 to 27 F_2 from cross C-I and 31 to 66 F_2 from C-II

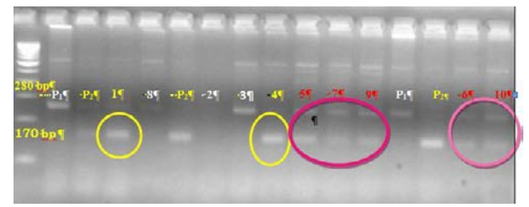

Fig. 2. PCR amplification pattern with Satt 409 primer in F_3 and F_4 generations, L Ladder Lane P_1 P. Kimya, P_2 NRC 102;2 F_3 From C-V; P_2 NRC 102 and 6 F_4 from C-VI, BC1 F_3 from C-I and C-II

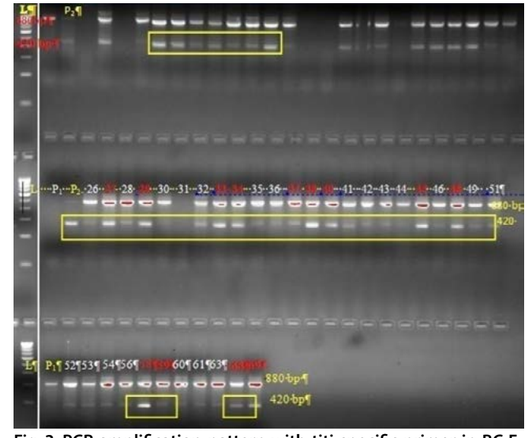

Fig. 3. PCR amplification pattern with titi specific primer in BC_1F_2 generation, L-Ladder; P_1:P. Agrani, P_2:NRC 101, #1 to #69 BC_1F_2 of C-I, C-II and C-V

Table 1. Segregating plant materials used for molecular study

S.No	Cross Name	F_2	F_3	F_4	BC_1F_2	BC_1F_3	BC_2F_3
1.	C-I (P.Agrani × NRC101)	8	—	—	18	6	2
2.	C-II (P.Agrani × NRC102)	8	—	—	18	6	3
3	C-III (P.Sangam × NRC 101)	11	—	—	4	—	—
4	C-IV (P.Sangam × NRC 102)	12	—	—	4	—	—
5	C-V (P.Kimya × NRC 101)	—	2	—	15	6	2
6	C- VI (P.Kimya × NRC 102)	—	—	6	—	—	—

33 and #34) and three plants from P. Kimya × NRC101 (#37, #58 and #59). Heterozygosity *Titi* with both *Satt409* 170bp and 280 bp markers were observed in 13 BC1F2 plants.

Eighteen BC1F3 and 7BC2F2 plant samples were amplified with *Satt409* primers. Among them 15 BC1F3 and 7BC2F2 showed amplification with 10 BC1F3 and 3BC2F2 plants amplifying a null allele linked 170 bp marker, while 4 BC1F3 and 3 BC2F2 amplified 280 bp marker. Heterozygosity was observed with both markers present in a BC1F3 and BC2F2 plant. Among those amplifying only *titi* null allele specific 170 bp marker included 6 plants from cross P. Agrani × NRC101 (#2, #3, #4, #5, #30 BC1F3 and #21 BC2F2 resp.), 3 from P. Agrani × NRC102 (#8, #9 and #10 BC1F3) and 4 from P. Kimya × NRC101 (#13, #14 BC1F3and #17, #18 BC2F2) (Fig. 5). It could be concluded that these10 BC1F3 and 3 BC2F2 generation plants had homozygous null (*titi*) genotype. Heterozygosity (*Titi)* was observed in a BC1F3 plant from cross P. Kimya × NRC 102 and in a BC2F2 plant from P. Agrani × NRC 102. The progeny derived from these plants must be screened in subsequent generations to select homozygous KTI free plants.

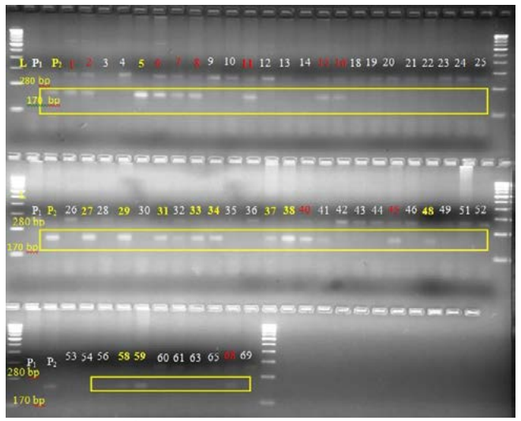

Fig. 4. PCR amplification pattern Satt 409 primer in BC_1F_2 generation, L-Ladder P1 P. Agrani P_2 NRC 101, #1 to #69 BC_1F_2 of C-I, C-II and C-V

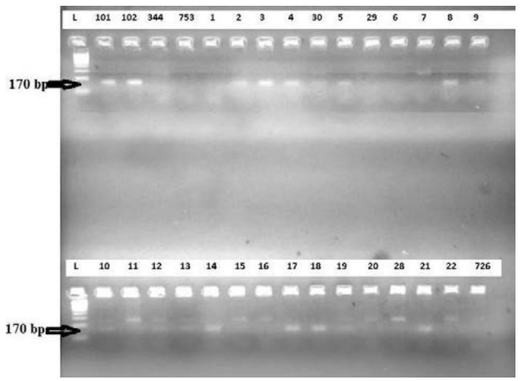

Fig. 5. PCR amplification pattern observed with SSR Satt 409 primer in BC1F3 and BC2F2, lane1; ladder, lane2; NRC 101, lane3; NRC 102, lane4; #P. Agrani/KDS 344, lane5; #P. Kimya/KDS 753, Lane32; P. Sangam/KDS726.; BC1F3: #1 to #5(CI), #29 to #10(C-II), #11 to #16 (C-V); BC2F2: #17 and #18(C-V), #19, #20 and #28(C-II), #21 and #22(C-I)

Kim et al. (2006) reported three SSR markers *viz*., Satt 228, Satt 409 and Satt 429 to be closely linked with the *KTI* gene while Moraes et al. (2006) had designed null allele *kti* specific *titi* primers. In the present study, null allele *kti* specific primer amplified 420 bp bands in null allele possessing plants that were either homozygous recessive (*titi*) or heterozygous (*Titi*) in BC1F2 generation; along with a monomorphic 880bp band. Satt 409 primers verified these results by amplifying 170 bp band in recessive (*titi*), both 170bp and 280bp in KTI expressing heterozygous *Titi* and 280 bp in KTI with *TiTi* genotypes. Overall, both null allele-specific *titi*-420bp marker and Satt409-170 together could identify 30 homozygous null (*titi*) plants *viz*., 2 F2; 1 F3,3F4, 11 BC1F2, 10 BC1F3 and 3 BC2F2. Simultaneously, heterozygous plants identified need to be screened in subsequent generation for selecting KTI free plants. Marker Assisted backcross breeding can help in developing KTI free soybean (Moraes et al. 2006; Rani et al. 2011; Kumar et al. 2013; Talukdar 1 et al. 2016). The *titi* null plants thus identified need to be further evaluated for their yield attributes.

Simultaneously trypsin inhibitor assay was undertaken to quantify the trypsin inhibitor present in the seeds of parents and their shortlisted progenies. Fine seed flour samples were defatted with petroleum ether, and the extraction of samples for the TI assay was performed as proposed by Kakade et al. (1974). A final centrifugation for 10 min at 3500 rpm allowed for the separation of the supernatant (extract) for the TI assay at 37°C (Bernard et al. 2017) and optical absorbance was recorded at 410 nm. In biochemical assay of trypsin activity, dark yellow colour was observed in sample without soymeal extract (Control) as well as on addition of soymeal extract from KTI free parents *viz*., NRC101 and NRC102, as well as from shortlisted twenty-eight KTI free lines *viz*. 2 F2,1 F3,1 F4,11 BC1F2,10 BC1F3 and 3 BC2F2. However, Phule parents did not show any color development due to the presence of trypsin inhibitors.

Trypsin inhibitor levels were high among the popular varieties (20.15 to 31.96 mg/g) as against null allele donor parents (3.02- 4.03 mg/g). TI content in the tentative KTI-free segregating populations was significantly lower (4.0 to 9.67 mg/g seed). The residual trypsin inhibitor activity (12.19-14.34%) in seeds was due to other trypsin inhibitors like Bowman Birk trypsin inhibitor, which is good both for human health and plant protection from pests as well as for prevention of pre-harvest sprouting. KTI free NRC101 and NRC102 recorded trypsin inhibitor activities of 4031 and 3023

TIU/g respectively; however, in the Phule varieties, higher KTI activity (20156. to 31967 TIU/g) was observed. The trypsin inhibitor content in the seeds of selected segregating plants ranged from 4031 to 8627 TIU/g, confirming absence of KTI in these plants. Inhibition of trypsin activity was higher (64.1-72.7%) among popular varieties as against null allele donor parents (10.7-14.3% inhibition) and the segregating plants selected (14.08- 28.72 % inhibition). Verma et al. (2015) analyzed 101 diverse soybean genotypes, with the Ti content in seeds ranging from 14.7 to 175.5 mg /g soy meal, while in Indian soybean varieties, it ranged from 58.8 to 126.8 mg/g. 'As per Shivakumar et al. (2015), trypsin inhibitor content in the Indian breeding lines generally ranged from 32 to 135 TI mg/g; while in the exotic collection, it ranged from 34 to 186mg/g. In the present study, Kunitz tripsin inhibitor (*Kiti null*) plant was identified using both molecular and biochemical studies may be useful for direct fortification or processing and animal feed industry.

Authors' contribution

Conceptualization of research (VA, MPD); Designing of the experiments (PC, MPD, AAK, RMN); Contribution of experimental materials (STP, RSB, MPD); Execution of field/lab experiments and data collection (STP, RSB, VPC, MPD); Analysis of data and interpretation (STP, RSB, VPC); Preparation of manuscript (STP, VPV, MPD).

Acknowledgement

The authors are thankful to the authorities of M.P.K.V., Rahuri, for providing the necessary facilities. The soybean genotypes with KTI null allele (NRC101 and NRC102) used in this work were provided by Dr. Vineet Kumar, ICAR-IISR, Indore. Technical help, provided by Dr. R.D. Sathbhai and Miss. R.C. Wadekar, Department of Biochemistry, M.P.K.V., Rahuri is also acknowledged.

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