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ST Pawale
Department of Agril Botany, and State Level Biotechnology Center, Mahatma Phule Krishi Vidyapeeth, Ahmednagar, Maharashtra, Rahuri, India

VP Chimote
Department of Agril Botany, and State Level Biotechnology Center, Mahatma Phule Krishi Vidyapeeth, Ahmednagar, Maharashtra, Rahuri, Maharashtra, India

MP Deshmukh
Department of Agril Botany, and State Level Biotechnology Center, Mahatma Phule Krishi Vidyapeeth, Ahmednagar, Maharashtra, Rahuri, Maharashtra, India

Corresponding Author:
ST Pawale
Department of Agril Botany, and State Level Biotechnology Center, Mahatma Phule Krishi Vidyapeeth, Ahmednagar, Maharashtra, Rahuri, Maharashtra, India

Accelerated development of Kunitz trypsin inhibitor free soybean genotype with charcoal rot resistance through marker assisted selection

ST Pawale, VP Chimote and MP Deshmukh

Abstract
Soybean [*Glycine max* (L) Merrill,] contains 38-44% Protein and 18-23% oil, which makes it unique among the cultivated crops. Presence of KTI is governed by a single dominant gene (*Ti*), while the absence is attributed to the recessive form (*titi*), which encodes for truncated protein. In soybean, there are ten independent differentially expressed *KTI* genes; of which KTI_3 is seed specific (Moraes *et al*., 2006) [8]. The recessive null *kti* allele contains one substitution and two deletions, which alters the translation process resulting in reduced levels of KTI in seed embryos (Jofuku *et al*., 1989) [5]. Kim *et al*., (2006) had reported that three SSR markers *viz*., Satt 228, Satt 409 and Satt 429 to be closely linked (0-10 cm) with the Kunitz trypsin inhibitor gene. After PCR amplification, the PCR products were resolved on 1% agarose gel electrophoresis for gene specific primer and 3% agarose gel electrophoresis for Satt 409 primer. Charcoal rot caused by (*Macrophomina phaseolina*) is the second largest yield reducing disease after brown spot (*Septoria glycines*) in the soybean growing countries across the globe. The fungus causes a general root rot in soybean, infecting the roots and lower stems. SSR Marker: Satt 640, Satt 542 and Satt 301 has been reported resistance to charcoal rot in segregating population. This disease reported under polygenic control. Heritability is also reported to be low. Charcoal rot is a disease that appears in hot and dry weather when soil temperatures are 80-90 °F (27-35 °F) for 2 to 3 weeks. The disease has been an endemic problem in relatively dry or drought-like situations.

Keywords: Soybean, null allele, Kunitz trypsin inhibitor, charcoal rot

Introduction
Soybean [*Glycine max* (L) Merrill,] contains 38-44% Protein and 18-23% oil, which makes it unique among the cultivated crops. Besides mineral and tocopherol, it contains flavonoids and other essential amino acids that help to keeping a good health of the regular consumers. The estimates of world soybean area, production and productivity for 2017-18 are 126.64 million ha, 346.31 million tons and 2.74 t/ha, as against the 2016-17 figures of 121.10 million ha, 348.85 million tons and 2.88 t/ha (Anonymous, 2017b).
Marker Assisted Selection (MAS) has been proved to be efficient in introgression of disease and insect resistance genes in several crops (Maroof *et al*., 2008 and Varshney *et al*., 2017) [7, 16]. MAS technique provides an efficient method for scoring nutritional quality traits (like protease inhibitor freeness or specific fatty acid profile etc.) which unlike morphological traits cannot be scored at field level by visual observation.
Presence of KTI is governed by a single dominant gene (*Ti*), while the absence is attributed to the recessive form (*titi*), which encodes for truncated protein. Genotypes with a null allele of KTI have been identified in soybean germplasm (Bernard *et al.,* 1991). Biochemically, KTI is a monomeric protein containing 181 amino acid residues (Roy Chaudhuri *et al*., 2003) [10]. In soybean, there are ten independent differentially expressed *KTI* genes; of which KTI_3 is seed specific (Moraes *et al*., 2006) [8]. Thirteen isozymes of KTI_3 protein encoded by multiple *KTI* alleles have been reported (Wang *et al*., 2012). The recessive null *kti* allele contains one substitution and two deletions, which alters the translation process resulting in reduced levels of KTI in seed embryos (Jofuku *et al*., 1989) [5].
The PI 542044, also known as 'Kunitz soybean', contains the null allele of KTI *i.e. kti* which encodes a truncated protein. This genotype was developed through backcrossing PI 157440 with a recurrent parent Williams 82; hence, it is considered as near isogenic line of Williams 82 (Bernard, *et al* 1991). It was developed at Agricultural Experimentation Station, University of Illinois and is a near-isogenic line of 'Williams 82', differing from the latter in the *KTi* genotype (Bernard *et al.,* 1991). This accession was procured from United States Department of Agriculture (USDA), however it failed to perform under Indian agro-climatic conditions. This accession exhibited poor agronomic performance with regards to germination and plant

architecture *viz*. height, branching and yield components. However, the absence of KTI in its seeds has been exploited in breeding programme focusing on development of food-grade soybean in India.

Gene for Kunitz trypsin inhibitor has been mapped and markers linked to the null allele have been reported (Kim *et al*., 2006; Rani *et al*., 2011a; Maranna *et al*., 2016) [6].

In this study Kunitz trypsin inhibitor *KTI* gene linked Satt 409-170bp and null allele (*titi)* specific 425bp markers were used to classify into recessive (*titi*), KTI expressing heterozygous carrier (*Titi)* and KTI expressing dominant (*TiTi*) alleles. Foreground selection approach using these two markers was used for selecting KTI free trait. SSR markers are valuable because they are codominant, highly polymorphic and are assayed efficiently by the PCR. Kim *et al*., (2006) had reported that three SSR markers *viz*., Satt 228, Satt 409 and Satt 429 to be closely linked (0-10 cm) with the Kunitz trypsin inhibitor gene; while Moraes *et al*. (2006) [8].

Recently, three recessive null alleles, *viz*., Kunitz trypsin inhibitor, soybean agglutinin, and P34 allergen nulls were stacked in the background of "Williams 82" and was termed as "Triple null" (Schmidt *et al*., 2015) [12]. Each of these nulls has the potential to partially address the concerns of soybean feed/food consumption. *Triple Null* has a slight reduction of total protein compared with 'Williams 82' corresponding to aggregate contribution of TI, LE and P 34 in the seed proteome. The 'Triple null' would have a potential application in conventional food and feed and for immunotherapy to mitigate soybean allergic responses (Schmidt *et al*., 2015) [12]. Production of soybean is highly challenged by abiotic and biotic stresses. Charcoal rot caused by (*Macrophomina phaseolina*) is the second largest yield reducing disease after brown spot (*Septoria glycines*) in the soybean growing countries across the globe. The fungus causes a general root rot in soybean, infecting the roots and lower stems. It was named from the fact that infected tissues look as if they have been dipped in charcoal dust. *M. phaseolina* infects an extremely wide range of hosts, including sorghum, soybean, cucurbits and various weed species. Its effect is more pronounced in crops under stress, biotic or abiotic. It is drawing more attention from the breeders in present time because of changing global climatic conditions. In fact, because of occurrence of frequent drought or drought-like situations is making soybean more vulnerable to this disease. Effect of the disease is not confined to the field alone, but it can affect the beans in storage conditions, as well. It can also reduce the seed germination badly.

Therefore, disease resistance to charcoal rot must be improved and incorporated into selected genotype to minimize yield loss. However, breeding for charcoal rot resistance through conventional technique did not met with much success. It is time consuming, laborious and largely ineffective. Hence, identification of molecular marker (s) linked to the charcoal rot resistance gene would greatly facilitate screening of breeding materials and thus accelerate the process of development of resistant cultivars. Here, advancement made in this respect at Indian Agricultural Research Institute, New Delhi is discussed.

Material and Methods

Table 1: Markers to be uses for screening of null (*kti*) allele

Sr. No.	Primer name	Forward sequence (5→3)	Reverse sequence (3→5)
01	Gene *titi* specific	CTTTTGTGCCTTCACCACCT	GAATTCATCATCAGAAACTCTA
02	Satt 409	CCTTAGACCATGAATGTCTCGAAGAA	CTTAAGGACACGTGGAAGATGACTAC
03	Satt 228	TCATAACGTAAGAGATGGTAAAACT	CATTATAAGAAAACGTGCTAAAAGAG

Table 2: PCR reaction details for the amplification of SSR primers

Adding sequence	Reagents	Stock concentration	Volume (µl)	Final concentration
1	Sterile Double Distilled Water	------	8.07	-
2	PCR Buffer with 15 mM $MgCl_2$	10X	1.5	1X (with 1.5mM $MgCl_2$)
3	$MgCl_2$ (extra)	15 mM	0.6	1.5 mM
4	dNTP Mix	10 mM mix	1.5	1.0 mM mix
5	Primer Forward	1µL	1.0	20 picomoles
6	Primer – Reverse	1µL	1.0	20 picomoles
7	Taq DNA Polymerase	3units/µl	0.33	1 unit
8	Template DNA	10ng/µl	1.0	
	Total Volume of Cocktail		**15**	

Molecular genotyping for null allele

Young (15 DAS) healthy leaves for DNA extraction were collect from individual plants and were immediately thawed in liquid nitrogen and then kept at -20 ºC for further use.

Table 3: PCR Thermal cycling conditions

Steps	Gene specific *titi* primer			Steps	SSR marker Satt 409		
	Temp (ºC)	Duration (Min)	Cycle (No)		Temp (ºC)	Duration (Min)	Cycle (No)
Initial denaturation	94 ºC	2	1	Initial denaturation	94 ºC	2	1
Denaturation	94 ºC	1	40 cycle	Denaturation	94 ºC	0.5	40 Cycles
Annealing	50 ºC	1		Annealing	50 ºC	0.5	
Extension	72 ºC	1		Extension	72 ºC	0.5	
Final Extension	72 ºC	10	1	Final Extension	72 ºC	10	1

Agarose Gel electrophoresis of PCR products

After PCR amplification, the PCR products were resolved on 1% agarose gel electrophoresis for gene specific primer and 3% agarose gel electrophoresis for Satt 409 primer.

Biochemical Assays

Table 3: Procedure for measuring TI activity by the continuous method

Sr. No.	Reagents	Control[a]	Sample[a]
1.	Tris buffer 0.050 MpH 8.2	1.00 ml	0.72 ml
2.	Trypsin working Solution	2.00 ml	2.00 ml
3.	Trypsin inhibitor extract	-	0.28 ml
Mix by inversion and equilibrate to 37 ºC for 2 min., then add BAPNA			
4.	BAPNA working solution (pre warmed at 37 ºC	1.30 ml	1.30 ml

The content of reaction mixture was mixed well and exactly after 10 minutes the data was recorded. The assay were performed at constant temperature of 37 ºC in water bath.

Absorbance measurement

Optical absorbance was recorded on spectrophotometer at 410 nm by using a thermostatic cuvette of 1 ml.

Calculation

The results were expressed in trypsin inhibitor units per gram of soybean flour (TIU/g) by continuous method.

$$\text{TIU/g} = \frac{100 \times 4.30 \times (M_{control} - M_{sample}) \times D \times 50.}{0.280}$$

$$\text{TI (mg g}^{-1}\text{ seed meal)} = \frac{\text{TIU/g}}{1000}$$

Screening of genotypes for charcoal rot resistance

The genotypes were subjected to screening for charcoal rot resistance in paper towel as per Nene *et al*. For this purpose, pure culture of the fungus was grown in PDA medium, incubated for 5 days at 25 ºC. The fungal mats were removed and macerated in 100ml sterile distilled water in which 5 days old seedling of test lines were dipped. 20-20 seedlings of each test line were kept in blotter paper and incubated at 25 ºC for ten days and were subjected for scoring. In each lot, one susceptible genotype was included to ascertain proper inoculation. The genotypes were observed periodically and watered occasionally. After 10 days of inoculation, scoring was done following 0-9 scale.

Markers used in segregating population for charcoal rot resistance

SSR Marker: Satt 640, Satt 542 and Satt 301.

Inheritance of charcoal rot

Complexities associated with resistant and susceptible genotype. No genotype found having immune or resistant complete. This disease reported under polygenic control and highly influenced by environmental conditions where the crop is grown. Heritability is also reported to be low in such condition selection is does not reported easily.

The genotype DT 974290 was released in USA during 2004 because of its high yield potential. Charcoal rot is very common in sorghum Deshpande *et al*. (2011). Study the genetics of charcoal rot resistance in *Rabi* sorghum was non-allelic interaction, dominance gene effect and dominance × dominance gene interaction was observed six parameter model of generation mean analysis. Duplicate gene interaction was observed for inheritance of charcoal rot resistance. Cross made between BAT 477 × A70 resistant and susceptible plant in the ratio of 9:7 resistances is controlled by two complementary gene interactions (Olya *et al*). Host of this disease occurred more than 500 plant spp. Pathogen is basidomycetes known in two amorph from belongs *microphomina* and *Rhizoctonia* diverse environmental condition saprophytic forms, Rhizoctonia bataticola mainly produce microsclerotia.

Macrophomina phaseolina is a non-specific manner and infect broad spectrum of economically important crops. Primary infection appear from the roots and ground level.

Symptoms

Clearly visible from the time of emergence and can be evaluated at various stages of plant growth generally cotyledons brown to black spots. Margins of cotyledons' becomes bright red, finally brown or black spot develop into large necrotic lesions and finally plant death occurs.

Disease cycle and manifeastation

Microsclerotia in the soil and on infected plant debris which serves as primary source of inoculum found persist in the soil upto three years. Released in the soil when the infected plant decayed. Germination occur throughout the season temperature requires at 28 to 35 °C and more microsclerotia occurred at low tissue water potential recognized as drought. *Macrophomina Phaseolina* produces slerotia in root which enable it to survive adverse environmental conditions. Microsclerotia in soil infected seeds or host tissue serve as primary inoculums.

Parental polymorphism

Parental polymorphism between resistant and susceptible genotypes were studied using SSR markers. The PCR products were resolving on 3% metaphor and scoring.

Hybrid purity testing of F_1 plants

Few sets of resistant and highly susceptible plants were crossed in different crossing in different combinations. F_1 seeds were harvested and grown in phytotron facility. DNA was extracted from tender leaves of the F_1 plants and was subjected to PCR amplification using polymorphic markers. True F_1 hybrid plants to produce heterozygous bands while selfed plants to produce bands like maternal plants. Seed only from the true hybrid plants were harvested to advance generation Talukdar *et al* (2009) [15].

Charcoal rot is a disease that appears in hot and dry weather when soil temperatures are 80-90 °F (27-35 °F) for 2 to 3 weeks. The disease has been an endemic problem in relatively dry or drought-like situations. The fungus is highly variable and can infect more than 500 crops and weed species. Its infection occurs in the spring when soil moisture is high; however symptoms of charcoal rot develop in the hottest, driest part of the growing season Talukdar *et al* (2009) [15].

Polymorphism study

In this study, polymorphism among the parental population was found to be nearly 50%. The number of alleles per marker was found to range from 2 to 4, only. It reflected the poor variability among the soybean genotypes used in the study.

Purity testing of F_1 hybrids and development of mapping populations

Identification of true hybrid plant is an important task in any breeding program. Conventionally, morphological differences between the putative hybrid plant and the parents are considered to identify the F_1 hybrid. However, result of such observation is often misleading.

Use of co-dominant molecular markers can offer solution to this problem. Here, the DNA from the F_1 plants was subjected to PCR amplification along with the parents using polymorphic SSR markers. True F_1 plants produced heterozygous bands while selfed plants produced bands like the maternal plants. Thus true F_1 hybrid plants were identified and seeds were harvest. This technique not only ensured accuracy in selecting true hybrid plants but also saved time, space and money.

Cultural management

Staggered sowing, crop rotation, reducing plant densities and irrigation 80% yield loss, reduce plant vigor and half filling of pods.

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