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Original scientific article

VALIDATION OF MOLECULAR MARKERS FOR THE KUNITZ TRYPSIN INHIBITOR PROTEIN IN SOYBEAN (*Glycine max* L. Merr.)

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Soybean (*Glycine max* L.) is a major source of plant proteins, but its use is restricted by anti-nutritional factors such as the Kunitz trypsin inhibitor (KTI). Eliminating KTI improves protein digestibility and reduces processing costs in food and feed production. This study aimed to use simple sequence repeat (SSR) markers in marker-assisted selection (MAS) for KTI-free soybean genotypes developed at the Maize Research Institute "Zemun Polje" (MRIZP). The new breeding line L003, derived from a cross between the standard line L584 and the KTI-free cultivar 'Laura,' was analyzed. Fifty-two F_8 seeds were screened with SSR markers Satt228 and Satt409, and the observed results were validated by detecting possible KTI protein by polyacrylamide gel electrophoresis (PAGE). In all L003 plants, homozygosity for the KTI-null genotype was observed by both SSR markers. Biochemical analysis showed the absence of the 21.5 kDa KTi protein band in L003, while the control genotype 'Lidija' with the dominant allele (*TiTi*) expressed KTI. Those results demonstrate a strong correlation between SSR marker profiles and the presence of KTI protein. Our results showed that both Satt228 and Satt409 are reliable tools for foreground selection in MAS, supporting the efficient development of KTI-free soybean cultivars. These results promote breeding strategies to develop nutritionally improved soybean varieties for food and feed industries without the necessity of thermal inactivating KTI.

Keywords: KTI, SSR markers, *Glycine max* L. Merr., MAS

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INTRODUCTION

The global increase in soybean production in Europe is the result of rising demands from the processing industry, as well as the European tendency to reduce its dependence on imports from other continents (PERIĆ *et al*., 2025; ILIN *et al*., 2025).

Soybean seeds are a rich source of protein, and their digestibility is a key indicator of dietary protein quality (WILSON, 2004; POPOVIĆ *et al*., 2013; VASILEVA *et al*., 2023). For protein-rich foods to contribute effectively to nutrition and health, their amino acids must be properly digested. However, physiologically mature soybean seeds contain several antinutritional factors that exhibit varying levels of biological activity. The primary protease inhibitors in soybeans are the Kunitz trypsin inhibitor (KTI) and the Bowman-Birk inhibitor (BBi), which together account for approximately 6% of the total soybean protein content (DIPIETRO and LIENER, 1989; PESIC *et al*., 2007). These antinutritional components, particularly KTI, inhibit trypsin and chymotrypsin, thereby impairing protein digestion by interfering with amino acid hydrolysis, with concentrations ranging from 2 to 6 mg/g (KUNITZ, 1947; ERDAW and BEYENE, 2018). KTI is a protein with a molecular weight of 21.5 kDa, composed of a single polypeptide chain of 181 amino acids stabilized by two disulfide bonds between cysteine residues (TAN-WILSON and WILSON, 1986). Feeding raw soybeans to monogastric animals is problematic, as the presence of KTI in animal feed has been associated with digestive disturbances, pancreatic hypertrophy, and liver damage (PALACIOS *et al*., 2004). At elevated temperatures, disulfide bonds are disrupted, leading to a reduction in KTI activity—a desirable effect that enhances protein digestibility.

According to ORF and HYMOWITZ (1979), the presence of KTI is governed by four forms of the *Ti* allele *(Ti a, Ti b, Ti c, and Ti d)* present in a codominant, multiple-allelic series at a single locus. Previous authors found that the fiffth form does not exhibit a soybean trypsin inhibitor and is inherited as a recessive allele designated *ti*. The monogenic nature of inheritance makes the selection for this trait more simplified than for the polygenic ones, implying the detection of recessive homozygotes in advanced generations of selfing via biochemical or molecular markers (PERIĆ *et al*., 2014).

Marker-assisted selection (MAS) is a molecular breeding technique that employs DNA markers, linkage maps, and genomic information to facilitate the development of improved plant cultivars. This approach enables breeders to enhance traits such as grain yield, quality, disease resistance, and environmental adaptability, thereby supporting the development of crop varieties that meet the demands of modern agriculture and sustainable food systems (COLLARD and MACKILL, 2008). In MAS, molecular markers closely linked to target genes are used to select genotypes within segregating populations that carry desirable traits. According to JOFUKU and GOLDBERG (1989), the KTI gene family includes at least ten distinct gene components, with many arranged in tandem pairs. Three of these families—KTI1, KTI2, and KTI3—were cloned and sequenced. For example, DNA markers Satt409 and Satt228 have been identified as closely linked to the *Ti* locus, with genetic distances of 4.5 cM and 0–3.7 cM, respectively, which controls the presence or absence of the KTI protein in soybeans (KIM *et al*., 2006; MORAES *et al*., 2006).

Soybean breeding program at the Maize Research Institute "Zemun Polje" (MRIZP) has developed genotypes lacking the Kunitz trypsin inhibitor, offering improved nutritional benefits through a combination of conventional and molecular breeding approaches. This study aimed to:

(1) screen newly developed line for the presence or absence of KTI using SSR markers, and (2) validate the effectiveness of these markers on soybean varieties developed at the Maize Research Institute "Zemun Polje".

MATERIALS AND METHODS

Experimental material consisted of 52 F_8 soybean plants randomly chosen from the advanced breeding line L003, developed from the cross of standard grain type line L584 and KTI-free cultivar Laura. Selected F_2 plants from this cross were advanced to F_5 by the pedigree method of selection. Among approximately 400 F_5 progenies, 45 were selected for field trials at multiple locations in two consecutive years, along with check varieties for the corresponding maturity group. Besides the grain yield, experimental lines in the field trials were evaluated for maturity, lodging, pod shattering, disease tolerance, plant height, 1000-seed weight, and seed quality. According to trials results, the line L003 has demonstrated superior agronomic performance for the mentioned traits and achieved a yield that meets the criteria for submission to the Commission for Variety Registration. Detection of the KTI in line L003 was performed in a sample of 52 randomly selected plants, by taking one grain per plant. Grains were processed using a Kataskapt mill to simultaneously grind the grains. The cultivar Laura (KTI-free) and breeding line L584 (containing KTI) were used as standard genotypes for the KTI presence/absence analysis.

DNA extraction and SSR genotyping

DNA was isolated from soybean grains using a commercial Qiagen DNeasy Plant Mini Kit (250) according to protocol. Two commercial SSR primers were used to check the presence of the KTi allele (Table 1). The reaction mixture for a PCR reaction consisted of 2.5 μL of reaction buffer (10x), 2 μL of nucleotide mix (10 mM), 0.2 μL of Taq polymerase, 0.25 μL of the forward (F) primer (0.5 μM) and 0.25 μL of the reverse (R) primer (0.5 μM). This mixture was made up to 19 μL with sterile, bidistilled water, and then 1 μL of the genomic DNA was added. The initial denaturation process took place at 94 ºC for 5 minutes, followed by 36 cycles of denaturation at 94 ºC, each lasting 30 seconds. This procedure involved primer binding through annealing temperatures for 45 seconds, followed by a final annealing step at 72 ºC for 1 minute. The final elongation phase lasted for 10 minutes at a temperature of 72 ºC. Amplified PCR products were separated on an 8% polyacrylamide gel using a 20 bp DNA ladder. Electrophoresis was performed on a Mini Protean Tetra-Cell (Bio-Rad) vertical polyacrylamide gel electrophoresis (PAGE) apparatus. After electrophoresis, the gels were photographed using a BDA Live System camera from Biometra.

Table 1. The set of KTI gene-specific markers used for PCR

Marker		Sequence
Satt228	F	5'-TCATAACGTAAGAGATGGTAAAACT-3'
	R	5'-CATTATAAGAAAACGTGCTAAAGAG-3'
Satt409	F	5'-CCTTAGACCATGAATGTCTCGAAGAA-3'
	R	5'-CTTAAGGACACGTGGAAGATGACTAC-3'

Protein extraction and electrophoresis

The extraction of KTI protein from soybean grain was conducted using the procedure outlined by HYMOWITZ and HADLEY (1972). Approximately 100 mg of ground samples were incubated in a buffer (0.092 M Tris-Cl pH 6.8, 0.023 M $CaCl_2$, and 0.5 mM PMSF) for 30 minutes at room temperature. Following the incubation, the samples were centrifuged and then mixed with a buffer containing 20% glycerol and 0.005% bromophenol blue. Electrophoresis was performed on a Mini Protean Tetra-Cell (Bio-Rad) vertical PAGE apparatus using a buffer composed of 25 mM Tris-HCl and 192 mM Glycine for electrophoresis. A Spectra Multicor low-range protein ladder (Thermo Scientific) and a BLUE wide-range protein ladder (Cleaver) were employed to identify samples lacking the Kunitz trypsin inhibitor. After staining with Imperial protein stain for one hour, the gels were photographed using a Nikon camera.

RESULTS AND DISCUSSIONS

Overcoming the challenge posed by anti-nutritional compounds in mature soybean seeds requires a detailed understanding of gene function and the application of advanced genetic tools in breeding. The development of KTI-free genotypes is of considerable importance in the food and feed industries, as it significantly reduces processing and heating costs. At the Maize Research Institute "Zemun Polje," several KTI-free soybean varieties have been developed (PERIC *et al*., 2014.). Molecular markers have become indispensable tools in modern plant breeding, enabling the efficient selection of desirable traits while conserving both time and resources (TANKSLEY *et al.*, 1989; NADEEME *et al.*, 2018). In this context, the microsatellite markers *Satt409* and *Satt228* have been successfully used to introgress favourable KTI alleles and to improve the efficiency of MAS for KTI-free genotypes.

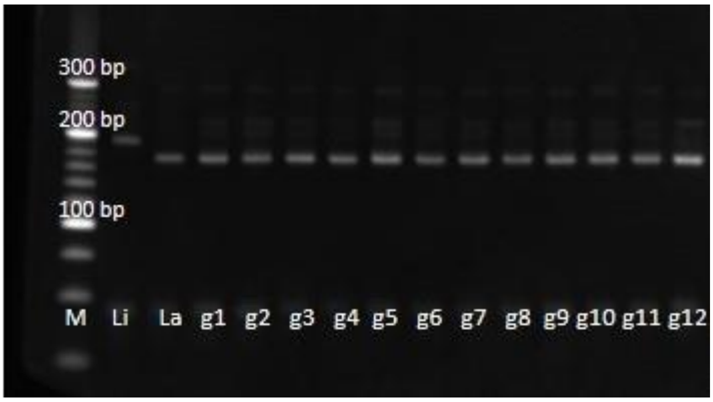

Figure 1. Electrophoregram of marker Satt409. M – 20 bp DNA ladder; Li - KTI variety Lidija; La - KTI free variety Laura. g1 – g12 F_8 seeds of line L003.

In this study, 52 F_8 individual soybean seeds from the new breeding line L003 were screened using the selected SSR markers (Figure 1). All 52 seeds were found to be homozygous recessive (**ti/ti**), consistent with the KTI-free allele present in the KTI-free cultivar 'Laura.'

Using the Satt409 marker, all seeds from the L003 line amplified the *ti* allele, approximately 165 bp in length, corresponding to the allele present in the donor parent, ‘Laura’. In contrast, the variety ‘Lidija’ amplified the dominant *Ti* allele associated with KTI presence, which was approximately 195 bp long (Figure 1). Similar results were obtained with the Satt228 marker: both L003 and ‘Laura’ amplified an allele of approximately 250 bp, while ‘Lidija’ displayed an allele of about 280 bp (Figure 2). These results are consistent with a previous study for the application of Satt228 marker in marker-assisted selection for KTI-free soybean genotypes developed in the MRIZP (MLADENOVIĆ DRINIĆ *et al*., 2011). Moreover, KOVAČEVIĆ *et al*. (2015) reported that the Satt409 marker amplified only the *TiTi* allele among 96 soybean varieties from the collection of MRIZP.

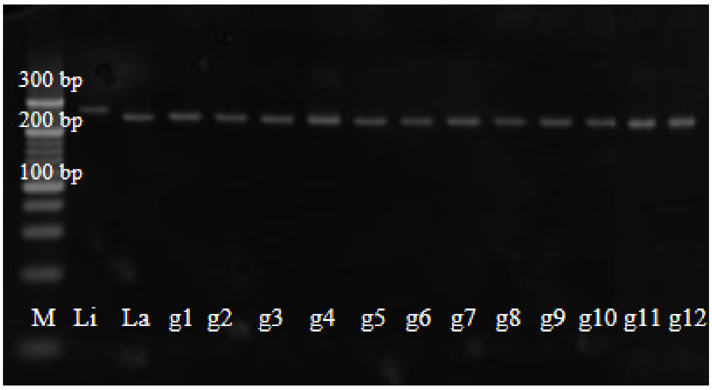

Figure 2. Electrophoregram of marker Satt228. M – 20 bp DNA ladder; Li - KTI variety Lidija; La - KTI free variety Laura. g1 – g12 F_8 seeds of line L003.

Our results are consistent with previous studies demonstrating that foreground selection for the KTIallele can be effectively performed using three closely linked SSR markers—Satt228, Satt409, and Satt429 (MARANNA *et al*., 2016). In that study, these markers were used for F_1 hybridity testing and foreground selection in BC_1 and BC_2 generations, producing polymorphic bands between the donor and both recurrent parents. Similarly, DHALIWAL *et al.* (2021), applied Satt409 to screen F_5 progeny derived from a cross between two varieties to confirm the presence of the null KTI allele. Using Satt228, heterozygous F_1 hybrids and homozygous titi lines were identified in F_2 segregating populations, enabling the development of potential titi homozygotes for breeding programs aimed at improving seed quality in Kazakhstan (BULATOVA *et al*., 2019). BUKAN *et al.* (2024) demonstrated that Satt228 effectively facilitated the early identification of KTI-free lines in F_2 progenies derived from crosses between low- and high-KTI genotypes.

To validate the molecular marker data, all genotypes were analyzed for KTI protein presence using polyacrylamide gel electrophoresis. Genotypes carrying the *TiTi* allele exhibited a clear protein band at 21.5 kDa, confirming KTI expression, whereas titi homozygotes lacked this band (Figure 3). Among the tested samples, only ‘Lidija’ displayed the 21.5 kDa band, fully corroborating the molecular results. Similar findings were reported by KOVAČEVIĆ *et al.* (2015), who demonstrated that Satt228 effectively identified KTI-free genotypes among 110 F_2 seeds, with titi lines lacking the 21.5 kDa band and displaying allele patterns corresponding to the absence of KTI.

A comparison of polyacrylamide gel electrophoresis profiles for the Kunitz trypsin inhibitor protein with the banding patterns generated by the Satt228 and Satt409 markers revealed a strong correlation. All *TiTi* genotypes exhibiting a 21.5 kDa protein band also displayed allele patterns amplified by Satt228 and Satt409 consistent with the presence of KTI. Conversely, all titi genotypes lacking the 21.5 kDa band showed marker profiles indicative of the absence of KTI. The combined use of SSR markers and biochemical confirmation of the KTI protein has been reported in earlier studies (RANI *et al.*, 2011; KUMAR *et al.*, 2015; BULATOVA *et al.*, 2019).

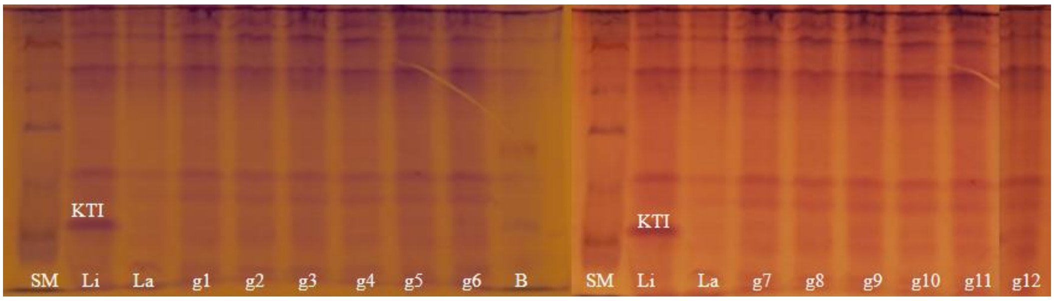

Figure 3. Biochemical confirmation of absence of KTI peptides. SM -Spectra Multicor low-range protein ladder; B- BLUE wide-range protein ladder; Li - KTI variety Lidija; La - KTI free variety Laura. g1 – g12 F_8 seeds of line L003.

Our findings confirm that both the Satt228 and Satt409 markers apeare to be effective tools for titi genotypes identification. Furthermore, they demonstrate that indirect selection using DNA markers closely linked to the *Ti* locus is a reliable approach for identifying all three genotypic classes (*TiTi*, *TiTi*, and *titi*). This strategy has significant advantages for breeding programs aimed at creating KTI-free soybean cultivars, as the co-dominant nature of the SSR markers allows for precise selection of favourable alleles, while MAS provides a simple and efficient means of accelerating the development of KTI-free soybean lines.

Latest research (PARK *et al*., 2023) identified a novel KTI-1 mutation and developed a corresponding new breeder-friendly KASP marker for selecting KTI-null plants. Re-sequencing of three soybean accessions without KTI-3 mutations revealed a unique three-base insertion and five missense mutations in the KTI.

CONCLUSIONS

The SSR markers linked to low KTI content, and validated in this study, showed a strong association with KTI levels in soybean varieties developed at the MRIZP. Both Satt409 and Satt228 proved reliable for detecting KTI allele variation and were instrumental in selecting progenies with reduced KTI content across segregating generations from crosses between parents with contrasting KTI genotypes and phenotypes. The application of MAS with these markers enabled the efficient development of KTI-free soybean varieties, suitable for producing soy-based foods and feeds without the need for thermal inactivation of KTI. Also, this study highlights the potential of MAS in accelerating the breeding process and reducing the cost and time associated with conventional selection methods.

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VALIDACIJA MOLEKULARNIH MARKERA ZA KUNITZ TRIPSIN INHIBITOR PROTEIN KOD SOJE (*Glycine max* L. Merr.)

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Izvod

Soja (*Glycine max L.*) predstavlja jedan od glavnih izvora proteina, ali njena upotreba je ograničena prisustvom antinutritivnih faktora kao što je Kunitz tripsin inhibitor (KTI). Eliminacija KTI poboljšava svarljivost proteina i smanjuje troškove prerade u proizvodnji hrane i stočne hrane. Cilj ovog istraživanja bio je korišćenje mikrosatelitskih (SSR) markera u selekciji pomoću molekularnih markera (MAS) za genotipove soje bez KTI, razvijene u Institutu za kukuruz „Zemun Polje“ (MRIZP). Analizirana je nova oplemenjivačka linija L003, dobijena ukrštanjem standardne linije L584 i sorte ‘Laura’ bez KTI. Pedeset dva F_8 semena linije L003 su analizirana pomoću markera Satt228 i Satt409, a rezultati su potvrđeni detekcijom KTI proteina poliakrilamidnom gel elektroforezom (PAGE). Kod svih biljaka linije L003 oba SSR markera su potvrdila homozigotnost za KTI. Biohemijska analiza pokazala je odsustvo proteinske trake od 21,5 kDa kod L003, dok je kontrolni genotip ‘Lidija’ sa dominantnim alelom (*TiTi*) ispoljio prisustvo KTI. Dobijeni rezultati ukazuju na snažnu korelaciju između SSR profila i prisustva KTI proteina, kao i korišćenje Satt228 i Satt409 markera kao pouzdanih alati za MAS u efikasnom razvijanju KTI-slobodnih sorti soje. takođe, navedeni rezultati unapređuju strategije oplemenjivanja usmerene na dobijanje nutritivno poboljšanih sorti soje za prehrambenu i stočarsku industriju, bez potrebe za termalnom inaktivacijom KTI.

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