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Identification of a Novel KTi-1 Allele Associated with Reduced Trypsin Inhibitor Activity in Soybean Accessions

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Abstract: Trypsin inhibitors (TIs) in soybean seeds reduce the availability of processed soybean foods and animal feed. This study aimed to evaluate the trypsin inhibitor activity (TIA) in 999 Korean soybean accessions and conduct molecular characterization of soybean accessions with low TIA. TIA was evaluated using colorimetric analysis through a substrate–enzyme reaction. The average TIA of the 999 soybean accessions was 90.31%, ranging from 43.70% to 99.51%. Kunitz trypsin inhibitor 3 (KTi-3) gene target sequencing analysis was performed on seven soybean germplasm accessions (IT105782, IT170889, IT273590, IT274513, IT274515, IT276197, and IT022891) showing less than 60% TIA. Four soybean accessions (IT274513, IT274515, IT276197, and IT022891) exhibited the same mutations (a G-to-T transversion and an AG deletion) in the KTi-3 gene (*Glyma.08g341500*) in PI542044. In addition, whole-genome re-sequencing was performed on three soybean accessions with no mutations in the KTi-3 gene. Compared with the reference soybean genome, an in-frame insertion and five missense mutations were identified in the coding sequencing of the KTi-1 gene (*Glyma.01g09500*) in IT105782. A RT-qPCR analysis showed that the mRNA expression level of KTi-1 was reduced by approximately 17% in IT105782 during seed development. In this study, we identified a previously unreported mutation in the KTi-1 gene and developed a KASP marker using this new KTi-1 variant.

Keywords: soybean; trypsin inhibitor (TI); trypsin inhibitor activity (TIA); KASP marker

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1. Introduction

Soybean seeds (*Glycine max* (L.) Merr.) are valuable sources of nutrition for both humans and animals. Because of the high demand for protein meals and vegetable oils, global soybean production is expected to increase by 6% year-on-year, reaching 361 million tonnes. Brazil (139 million tonnes) and the United States (120.8 million tonnes) were the world's largest soybean producers in 2021–2022 [1]. Soybean seeds comprise approximately 40% protein, 30% carbohydrates, and 20% fat, with the remaining 10% containing various functional substances [2]. In addition, soybeans contain non-nutritive elements such as isoflavones, which reduce blood cholesterol levels and increase bone strength [3]. Because

of their health benefits, soybeans are widely consumed in various processed forms, such as soybean paste, soy sauce, tofu, and soy milk, in Korea, China, and Japan.

However, soybeans also contain anti-nutritional elements such as lipoxygenase, tannins, trypsin inhibitor (TI), and phytates [4,5]. Especially, TI interferes with the amino acid hydrolysis reactions of trypsin and chymotrypsin, strongly inhibiting protein digestion [6]. Therefore, the presence of TI in animal feed causes digestive disturbances, pancreatic enlargement, and liver damage in monogastric animals [7–9]. TI makes up about 6% of the total soybean protein and can be divided into two major types: the Kunitz trypsin inhibitor (KTi) and the Bowman–Brik inhibitor (BBi) [10]. In the context of trypsin inhibition, KTi plays a more substantial role compared to BBi [11,12]. When evaluating trypsin inhibition on an equal-mass basis, KTi exerts a more pronounced influence than BBi [13]. This suggests that KTi exhibits specificity in TI. KTi is present in higher concentrations than BBi, and KTi content varies widely among different soybean genotypes, ranging from 4.28 to 15.0 mg per gram of soy flour [14,15]. Soybean KTi is a protein with a 21 kDa molecular weight that exists as a monomer, consisting of 181 amino acids linked by two disulfide cysteine residues, while BBi is a low-molecular-weight protein (7 kDa) consisting of 71 amino acids with seven disulfide cysteine residues [16,17]. The disulfide bonds in TIs increase their stability at high temperatures and decrease the availability of soybean processed foods [18]. To decrease the activity of TIs, high-temperature treatment at 100 °C for 40 min is sufficient, but it causes nutrient denaturation and requires significant effort [18].

Three genes (KTi-1, KTi-2, and KTi-3) were cloned from developing soybean seeds [19,20]. The KTi-1 gene (*Glyma.01g095000*) is located on chromosome 1, while the KTi-3 gene (*Glyma.08g341500*) is located on chromosome 8. The KTi-2 gene, on the other hand, is not included in the most recent soybean genome annotation [21]. The KTi-1 and KTi-3 genes are composed of a single CDS domain and exhibit about 80% sequence homology [20].

The mRNA expression of KTi is mainly observed in the endosperm and cotyledon of seedlings, with rare occurrences in other regions [20]. In soybean seeds, the expression level of KTi protein is first detected at a seed length of 3–4 mm and reaches its peak in 11–12 mm seeds, gradually decreasing thereafter [22]. The KTi-3 gene accounts for more than 80% of total KTi expression [23].

Several soybean accessions with low KTi activity have been found in soybean germplasm. According to Orf and Hymowitz, KTi activity is reduced by 40% to 50% in PI157440 and PI196168 in the USDA homogeneous germplasm collection [24]. These two accessions contain the same mutations (one G-to-T transversion and two nucleotide sequence deletions) in the KTi-3 gene, resulting in a truncated protein [25]. In addition, Gillman et al. (2015) identified PI68679 with reduced KTi content among 520 soybean accessions in the USDA soybean germplasm collection. PI68679 has a single-base deletion in the KTi-1 gene (GGG356 → GG358), resulting in a frameshift mutation [21]. The KTi-1 null allele shows a reduction in trypsin inhibitor activity (TIA) similar to that of the KTi-3 null allele, and the combination of the KTi-1 and KTi-3 null alleles results in a greater reduction in TIA [21]. Recently, a low KTi genotype, Punjab1, was identified in Indian soybean germplasm [14]. However, the specific genetic variation contributing to the low KTi phenotype in Punjab1 has not yet been determined.

Several molecular markers have been developed to assist in the selection of null-KTi soybean lines without the need for biochemical analysis [26,27]. Since Kim et al. [28] identified three simple sequence repeat (SSR) markers (Satt228, Satt409, and Satt429) closely linked to the KTi-3 null allele within a range of 0–10 cM, these SSR markers have been broadly used to introgress the KTi-3 null allele in soybean breeding programs [26,27]. Recently, the single-nucleotide polymorphism (SNP) loci of the KTi-3 null allele have been employed to develop functional markers based on high-throughput and automated SNP genotyping platforms such as the Simple Probe assay and the Kompetitive allele-specific polymerase chain reaction (KASP) genotyping assay [21,29].

In general, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) has been utilized to identify KTi in soybeans [30]. On the other hand, the Lab-on-a-Chip,

ELISA, two-dimensional liquid chromatography, and high-performance liquid chromatography (HPLC) methods have been developed to quantify KTi [21,31–34]. However, these methods need specialized equipment. Currently, a colorimetric enzyme assay based on the substrate–enzyme reaction mode is widely used for large-scale analysis [25,35]. $_{DL}$-BAPA (Na-Benzoyl-D, L-arginine 4-nitroanilide hydrochloride) is used as a synthetic substrate, and the bovine trypsin enzyme cleaves it to release a yellow-colored p-Nitroaniline, which shows strong UV/V absorption at 416 nm [35]. A high amount of TI in the sample extract reduces TI, leading to a decrease in p-Nitroaniline, while a low amount of TI in the sample extract leads to an increase in p-Nitroaniline [35]. Substrate–enzyme reaction methods are classified into the E-last test and the S-last test. The E-last test involves reacting the inhibitor and the substrate first and then adding the enzyme to confirm the colorimetric reaction. In contrast, the S-last test involves reacting the inhibitor and the enzyme first and then adding the substrate [36]. The stability of the experimental results differs depending on the order in which the substrate and the enzyme bind to the TI. In a recent study, it was confirmed that the E-last test shows consistent results between pH 3.0 and pH 9.0, while the S-last test shows higher-than-average measured values above pH 7.0 [37].

In this study, we adapted Liu's method to analyze 999 soybean accessions rapidly at a low cost, with the goal of screening for low TIA and identifying novel KTi-null mutations for the development of molecular markers [37]. We successfully screened low TIA, identified the KTi-1 mutation, and developed a KASP marker. The newly developed screening marker is efficient in selecting for low TIA traits, which will aid in the breeding of KTi-null soybeans in the future.

2. Materials and Methods

2.1. Plant Materials

In this study, a total of 999 soybean accessions were obtained from the Genebank of the National Agrobiodiversity Center at the Rural Development Administration (RDA) in the Republic of Korea. All accessions were grown in 2019 in the experimental field of the RDA Genebank in Jeonju, Republic of Korea (located at 35°49′53″ N, 127°03′50″ E). After sowing two plants per hole in a 90 cm × 15 cm spaced plot, one plant was removed, and three plants were harvested. We acquired 30 seeds for each accession from the Genebank, all of which were harvested in 2019, and used them directly for TIA assay. The controls consisted of two germplasms: Daepung 2 (IT269977) (KTi-1 and KTi-3) as the wild type and PI542044 with the null KTi-3 mutation. PI542044, a soybean accession with a null KTi-3 allele, was created through five backcrossing generations involving 'Williams 82' and PI157440 at both the Illinois Agricultural Experimentation Station and the USDA-ARS in the USA. PI542044 was obtained from the USDA Germplasm Resources Information Network (GRIN) (http://www.ars-grin.gov, accessed on 26 October 2023). On 21 June 2021, one wild-type soybean, null KTi-3 soybean and eight low-TIA soybean accessions were planted in Chonnam National University Field (35°10′32″ N, 126°54′25″ E). Two plants were placed in one hole at 20 cm intervals on 0.7 m × 1.5 m plots. These plants were used for DNA extraction, RNA extraction, and agronomic trait evaluation.

2.2. TIA Assay

This experiment modified Keshun Liu's TIA to test 15 samples simultaneously in three replications, streamlining large-scale analysis with minimal resource utilization [37]. The changes included reducing the volume to 1 mL and performing the reactions in a dry bath at a constant temperature of 40 °C. In this experiment, 10 mg of seed powder, sieved using a 500 µm mesh sieve, was mixed with 1.5 mL of 10 mM NaOH buffer. The protein extract was obtained after centrifugation at 10,000 rpm for 30 min. In a 2 mL deep-well plate, a specified volume of 100 mM Tris-HCl buffer (pH 8.2, containing 20 mM $CaCl_2$), protein extract, and 40 °C BAPA Working Solution were added and allowed to react for 10 min. Trypsin solution was added to react at 40 °C for 20 min, and the reaction was stopped with a 30% acetic acid solution.

2.3. Calculation of TIA

The TIA (%) was calculated by measuring at 410 nm after the completion of the reaction. The data were displayed as the average ± standard deviation (SD). TIA indicates the activity of TI as a percentage. To obtain TIA (%), absorbance values for four conditions (Reference, Reference Blank, Sample, and Sample Blank) were calculated and substituted into the formula below [38,39]. TIA was measured in triplicate, and absorbance was evaluated at 410 nm using an Epoch Microplate Spectrometer (BioTek instrument, Inc. Winooski, VT, USA).

$$TIA(\%) = \frac{[(A_R - A_{RB}) - (A_s - A_{SB})]}{(A_R - A_{RB})} \times 100(\%)$$

A_R, absorbance of Reference;
A_{RB}, absorbance of Reference Blank;
A_S, absorbance of Sample;
A_{SB}, absorbance of Sample Blank.

2.4. DNA Extraction and Kompetitive Allele-Specific PCR (KASP) Assay

Fresh leaves were harvested from one wild-type soybean plant and eight low-TIA soybean plants in 2021. DNA was isolated using a DNeasy Plant Kit (Qiagen, Hilden, Germany), and its concentration and purity were analyzed using a Nanodrop ND 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). KASP primers were designed using candidate gene sequences to distinguish the types of KTis in soybean mutants (Table 1). KASP assays were conducted in a final reaction volume of 10 µL, consisting of 5 µL of KASP master mix, 0.14 µL of primer mix, and 5 µL of 50 ng/µL DNA. The KASP PCR cycling involved an initial pre-reading cycle at 30 °C for 1 min, a holding stage at 94 °C for 15 min, 10 touchdown cycles at 94 °C for 20 s starting from 61 °C and reducing by 0.6 °C per cycle down to 55 °C for 1 min, 35 cycles at 94 °C for 20 s and 55 °C for 1 min, and a post-PCR reading stage at 30 °C for 1 min, with subsequent analysis of end-point fluorescence data using StepOne software (Applied Biosystems).

Table 1. List of primers used in KASP.

Marker	Gene ID (Glyma.Wm82.gnm2)	Fluorescent Primer	Sequence
KTi-1	*Glyma01g095000*	FAM_primer	CTACTGTATCGCGTGCAGCAAGTT
		HEX_primer	TACTGTATCGCGTGCAGCAAGTG
		Common reverse primer	GAGAGAGAGGGTCTACAAGCTGTTA
KTi-3	*Glyma08g341500*	FAM_primer	TGCAATGGATGGTTGGTTTAGACTTG
		HEX_primer	ATGCAATGGATGGTTGGTTTAGACTTT
		Common reverse primer	CTGTGGACAGAACACAAGCTTATAGTTAT

2.5. Whole-Genome Re-Sequencing

Re-sequencing analysis was performed on low-TIA soybeans for which no mutations were found by target gene sequencing. Using an Illumina DNA library preparation kit, we constructed three low-TIA soybean sequencing libraries and sequenced them on an Illumina NovaSeq 6000 platform. The trimmed reads were aligned to the soybean reference genome [40] using the BWA-MEM [41], resulting in the generation of a SAM format file for SNP analysis. After filtering, the SNP matrix was generated using the SAM tools (v.0.1.16) [42]. SNP annotation was performed based on the genetic location information of the soybean reference genome. Reads with over 90% identity were categorized as "homozygous SNPs"; those with similarity ranging from 40% to 60% were classified as "heterozygous SNPs". In addition, "N" was utilized to classify mixed cases, while "n" was utilized to indicate cases where read mapping was omitted.

2.6. RT-qPCR Assay

Total RNA was isolated from two seed stages: mid maturation (200–300 mg, seed fresh weight) and late maturation (400–500 mg, seed fresh weight). Total RNA extraction was conducted as per the manufacturer's guidelines using the Ribospin^{TM} Plant (GeneAll, Republic of Korea). RNA quality was determined by measuring the absorbance at 260 nm using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA), and the RNA concentration of the samples was adjusted to 50 ng/µL. Complementary DNA (cDNA) synthesis was carried out with the SuperScript^{TM} III First-strand Synthesis SuperMix kit (Invitrogen, Carlsbad, CA, USA).

Quantitative PCR was performed on the ABI StepOnePlus system (Applied Biosystems, Waltham, MA, USA) using iQTM SYBR Green Supermix (Bio-RAD). The qPCR reaction was carried out with 2 µL of cDNA (100 ng), 10 µL of cDNA (100 ng), 10 µL of 2X SYBR® Green Supermix, 3 µL of KTi-1 primer (Table 2; 300 nM), and 3 µL of D.W. in a total reaction volume of 20 µL. Quantitative PCR assays were performed under the following conditions: preheating for 1 min at 95 °C, denaturation for 3 min at 95 °C, and 40 cycles of incubation at 95 °C for 15 s and at 60 °C for 1 min for amplification. Following that, a melting curve analysis was carried out to validate both the absence of a product and the formation of dimers among the primers. The relative expression levels of target genes were calculated using the previously reported $2^{-\Delta\Delta CT}$ method [40], and cons7 (insulin-degrading enzyme, metalloprotease) was used as a housekeeping gene [43,44].

Table 2. List of primer sequences used for target sequencing and RT-qPCR.

Gene	Gene ID (Glyma.Wm82.gnm2)	Forward Primer (5′ → 3′)	Reverse Primer (5′ → 3′)	Product Size	Purpose
KTi-1	*Glyma01g09500*	TGGAGAGAGAGGGTCTACAAGC	TTAGACAGCACTAAACGCCTGA	196 bp	RT-qPCR
cons7	None	ATGAATGACGGTTCCCATGTA	GGCATTAAGGCAGCTCACTCT	114 bp	RT-qPCR

2.7. Evaluation of Agronomic Traits

We investigated the agronomic traits of three accessions that did not have the KTi-3 mutation. In 2021, agronomic trait evaluations were conducted for three accessions, following the criteria introduced by the RDA's GenBank for soybean genetic resource characteristic evaluations. These evaluations aimed to assess various agronomic traits, such as plant height, number of branches, number of nodes, pod count, seed yield per pod, 100-seed weight, flowering date, and maturity date. Each evaluation was repeated five times to ensure accuracy.

2.8. Statistical Analysis

The statistical analyses were conducted using IBM SPSS Statistics 27 (IBM, Armonk, NY, USA) [45] Student's *t*-tests were employed to compare the impact of genotypes on gene expression. The *t*-test results were indicated with an asterisk (*) for a significant difference with $p < 0.05$, with two asterisks (**) for a significant difference with $p < 0.01$, and with "ns" for non-significant differences. Agronomic trait results were analyzed using a one-way analysis of variance (ANOVA) and the Duncan test.

3. Results

3.1. Analysis of TIA in 999 Soybean Accessions

A substrate enzyme analysis was performed to measure the activity of TIs in 999 soybean accessions. The TIA values for these accessions ranged from 43.70% to 99.51%, with an average of 90.31% (Figure 1 and Table S1). The majority of soybean accessions (625) exhibited a high level of TIA (>90%), while 263 accessions showed a moderate level of TIA (80–90%). However, seven soybean accessions, namely, IT274515, IT273590, IT274513, IT170889, IT276197, IT022891, and IT105782, showed less than 70% TIA (Table 3). Among the 999 soybean germplasms, the lowest TIA was detected in IT274515. In addition, five

accessions, excluding IT022891 and IT105782, exhibited lower TIA levels compared to PI542044 (null KTi-3).

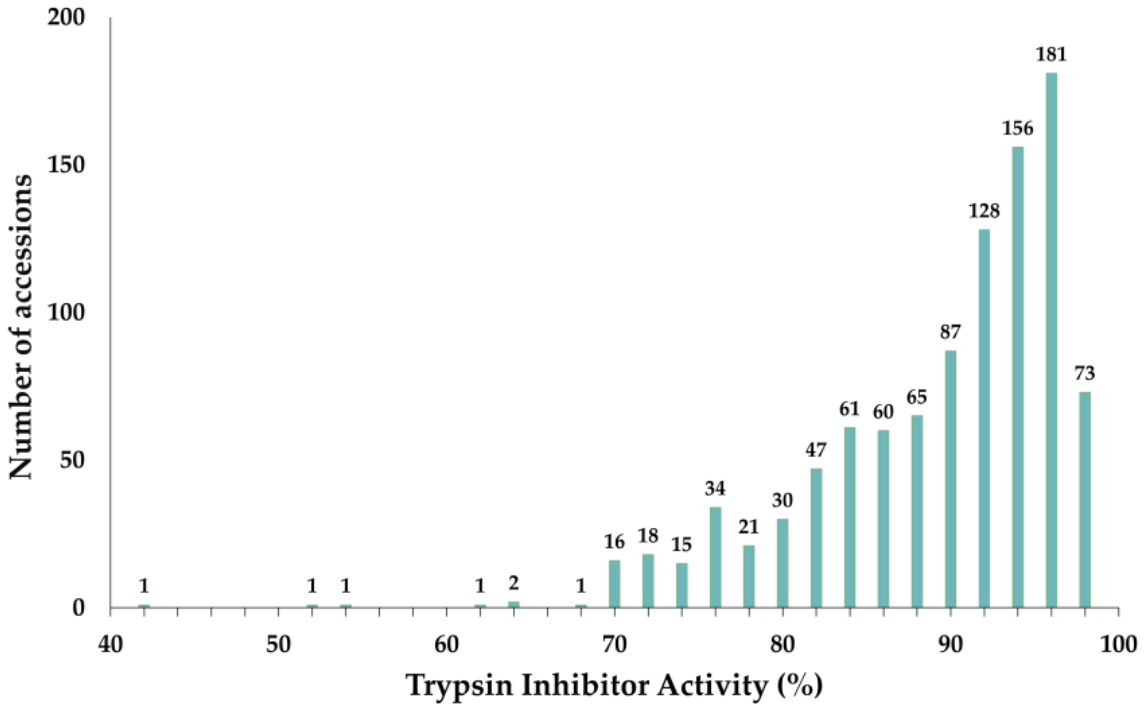


Figure 1. Distribution of trypsin inhibitor activity (TIA) in 999 soybean germplasms.

Table 3. Trypsin inhibitor activity (TIA) percentage values in seven soybean accessions and reference soybeans (mean ± standard deviation, $n = 3$).

Accession Name	Genotype Name	TIA (%)	Origin
IT274515	014502	43.70 ± 0.21	KOR
IT273590	MNG-PSARI-1998-11	53.10 ± 2.67	KOR
IT274513	014499	54.50 ± 4.38	KOR
IT170889	Hood 75	62.90 ± 0.57	USA
IT276197	GSI 014099	64.15 ± 5.71	Unknown
IT022891	PI 74866	64.75 ± 1.34	Unknown
IT105782	Kongnamul Kong	68.98 ± 1.89	KOR
IT269977	Daepung 2 (normal KTi)	89.15 ± 0.22	KOR
PI542044	Kunitz (null KTi-3)	66.60 ± 1.41	USA

3.2. Results of KASP Assay Screening for KTi-3 Allele

A comparison of the base sequences between Daepung 2 and PI542044 confirmed the presence of two mutations: the 436G → T missense mutation and the 440GAG → G frameshift mutation. Based on the null KTi-3 mutation found in PI542044, we developed a KASP marker targeting the 436G → T mutation in the KTI-3 gene. To distinguish KTi-3 mutations, we analyzed seven accessions, including the KTI-null PI542044 and the wild type Daepung 2. The analysis showed that IT274513, IT274515, IT276197, and IT02289 had the same mutation as PI542044 (Figure 2). Furthermore, Daepung 2, IT105782, IT170889, and IT273590 were identified as having the wild-type allele in the KTi-3 gene.

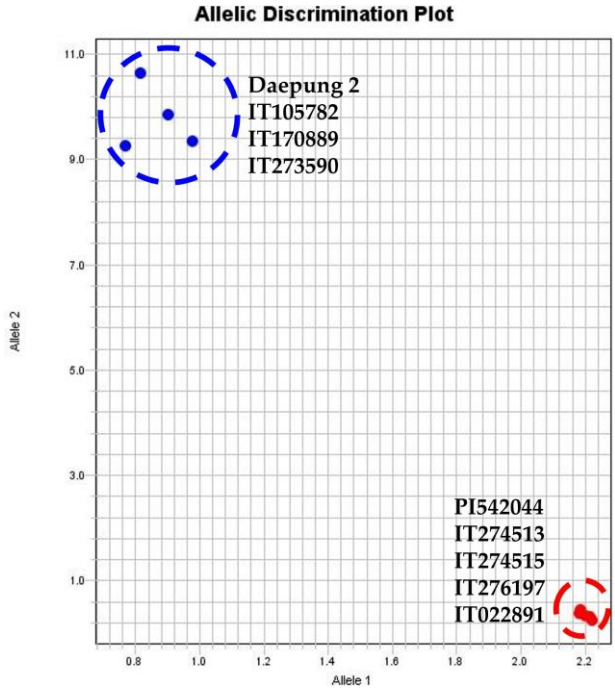


Figure 2. KASP assay screening for KTi-3 using HEX and FAM fluorescent fluorophores. Shown in red are the soybean lines with KTi-3 mutation presented by HEX fluorescence, and in blue are the soybean lines with normal KTi-3 allele presented by FAM fluorescence.

3.3. Whole-Genome Re-Sequencing

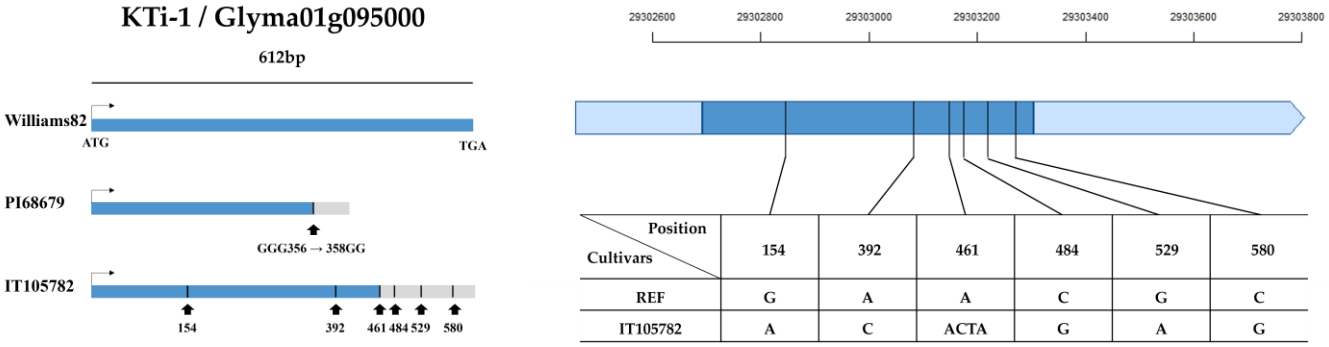
A resequencing analysis was performed on three soybean accessions (IT105782, IT170889, and IT273590) that showed no KTi-3 gene mutations in the KASP analysis. Genome sequencing of the three accessions generated over 587.86 million reads (88.77 Gb), with an average of 195.96 million reads (29.59 Gb) per accession (Table 4). After the cleaning step, 95.75% to 95.81% of the reads were mapped to the reference soybean genome. On average, each soybean accession covered 95.79% of the reference genome. The mean sequencing depth ranged from 26.32× in IT273590 to 27.38× in IT170889, with an average of 26.90×. A comparison of the three soybean accessions with the reference sequence revealed a total of 5,619,625 variants, including 4,649,462 SNPs and 970,163 indels. Specifically, IT105782 had 1,583,974 SNPs, 333,575 indels, and 1,917,549 total variations. Furthermore, IT170889 had 1,561,108 SNPs and 320,010 indels, while IT273590 had 1,504,380 SNPs and 316,578 indels.

Table 4. Summary of genome re-sequencing and DNA sequence variants identified in three soybean accessions with low Kunitz trypsin inhibitor (KTi) activity.

	IT105782	IT170889	IT273590	Mean	Total
Total reads	196,617,960	199,490,702	191,750,912	195,953,191	587,859,574
Average sequence length (bp)	151	151	151	151	151
Total size	29,689,311,960	30,123,096,002	28,954,387,712	29,588,931,891	88,766,795,674
Total size (Mb)	29,689	30,123	28,954	29,588.7	88,766
Sequencing depth (×)	26.99	27.38	26.32	26.90	-
Mapped reads	186,915,887	189,267,121	182,758,171	186,313,726	558,941,179
Genome coverage (%)	95.81%	95.75%	95.80%	95.79%	-
Number of SNPs	1,583,974	1,561,108	1,504,380	1,549,821	4,649,462
Number of indels	333,575	320,010	316,578	323,388	970,163
Total variation	1,917,549	1,881,118	1,820,958	1,873,208	5,619,625

3.4. Novel Allele in KTi-1 Gene

Based on the NGS data, no significant variations were found in the KTi genes of IT170889 and IT273590. However, we found that IT105782 had five base-substitution mutations and an in-frame insertion of 3 bp in the KTi-1 gene (Figures 3 and S1). The five nucleotide substitutions resulted in missense mutations, specifically causing the alterations G → A at position 154, changing Gly to Ser; A → C at position 392, changing Lys to Thr; C → G at position 484, changing Gln to Glu; G → A at position 529, changing Asp to Asn; and C → G at position 580, changing Gln to Glu (Figure S2). These missense mutations were all non-conservative. Additionally, an in-frame insertion of CTA at position 461 resulted in the generation of tyrosine. Overall, the results of genome sequencing indicate that the KTi-1 mutations in IT105782 were different from those in PI68679 (GGG356 → GG358, relative to the start codon; Figure 3A).



(**A**) (**B**)

Figure 3. (**A**) Overall visual description of the reference sequence, KTi-1 mutation, and the novel KTi-1 mutation. (**B**) Detailed visual description of the novel KTi-1 mutation.

3.5. Analysis of mRNA Expression Levels of KTi-1 Genes

To evaluate the role of the KTi-1 gene in soybean seeds, we analyzed its expression levels at two different stages of seed development and compared them to both IT105782 and the wild type genotype. The analysis revealed a significant decrease in the expression of the KTi-1 gene in IT105782 (a KTi-1 mutant) during both the mid- and late stages (Figure 4). In the mid-stage, IT105782 showed a significant decrease of 63% compared to Daepung 2. Additionally, a significant decrease of 17% was observed in the late stage compared to Daepung 2.

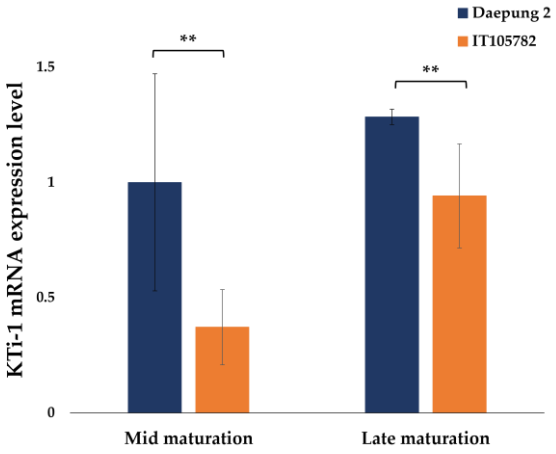


Figure 4. KTi-1 mRNA expression levels of three soybean accessions with low Kunitz trypsin inhibitor (KTi) activity. **, $p < 0.01$.

3.6. Detection of Mutations in KTi-1 Candidate Genes Using KASP Markers

The KASP marker was based on the KTi-1 variant candidate gene *Glyma01g095000* and was designed using the missense mutation at location 392. The newly developed KASP marker effectively distinguished between the KTi-1 mutant and the non-variant alleles (Figure 5).

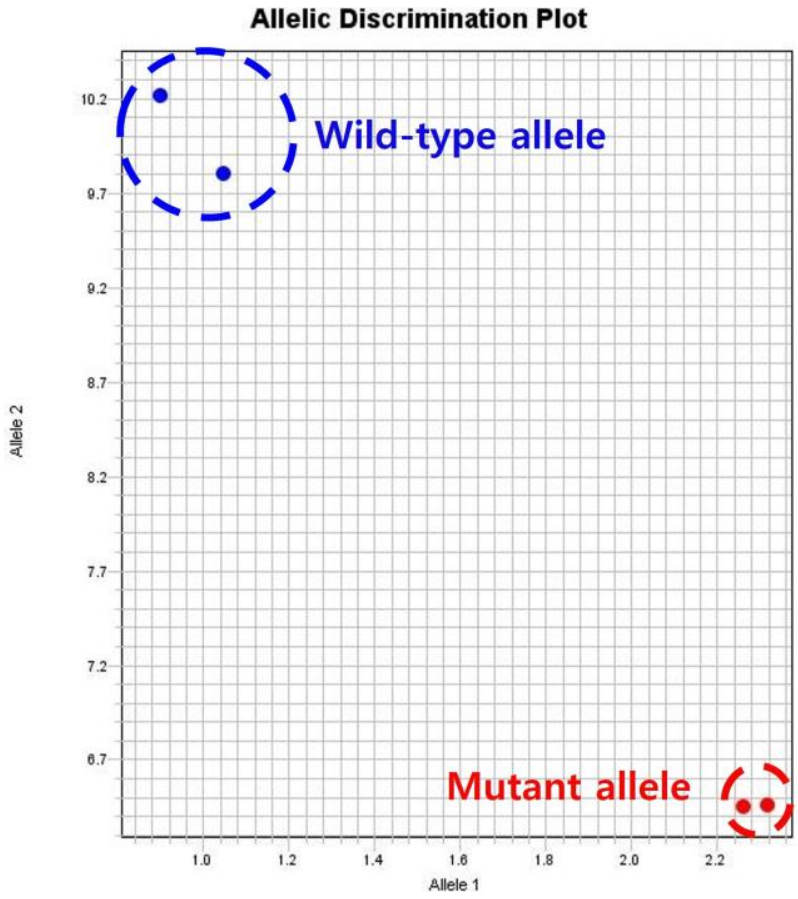


Figure 5. KASP assay for KTi-1 using HEX and FAM fluorescent fluorophores. Shown in red are the soybean lines with KTi-1 mutation concentration accumulation, indicated by HEX fluorescence, and in blue are the differences from KTi-1 cultivars, indicated by FAM fluorescence.

3.7. Evaluation of Agronomic Traits

The agricultural characteristics of four soybean accessions harvested in 2021, namely, IT105782, IT170889, IT273590, and Daepung 2, were evaluated. As shown in Figure 6 and Table 5, the three soybean accessions (IT105782, IT170889, and IT273590) exhibited higher plant height compared to Daepung 2, accompanied by more pods and branches. Daepung 2 showed a flowering date that was 10.6 days earlier than the average of the three accessions. IT105782 showed the quickest maturation, while IT273590 exhibited the slowest. The average 100-seed weight (16.9 g) of the three soybean accessions was half that of Daepung 2 (30.88 g), and the seed numbers per pod were lower than that of Daepung 2. In general, the agronomic traits of the three soybean accessions were deemed unsuitable for use as soybean cultivars.

Figure 6. Plants (**A**) and seeds (**B**) of three soybean accessions with low Kunitz trypsin inhibitor (KTi) activity.

Table 5. Major agronomic traits of three soybean accessions with low Kunitz trypsin inhibitor (KTi) activity and the control cultivar.

Cultivar	Plant Height (cm)	Number of Branches	Number of Nodes	Number of Pods	Seeds per Pod	100-Seed Weight (g)	Flowering Date	Maturity Date
Daepung 2	50.8 ± 3.27 ^{a†}	2.6 ± 0.55 ^{a}	12.4 ± 2.07 ^{a}	57.0 ± 10.44 ^{a}	3	30.9 ± 1.60 ^{d}	29 July	27 October
IT105782	70.0 ± 5.05 ^{b}	11.0 ± 1.87 ^{b}	15.4 ± 1.14 ^{b}	120.4 ± 14.84 ^{b}	2	12.7 ± 0.74 ^{a}	2 August	15 October
IT170889	88.6 ± 1.34 ^{c}	13.0 ± 2.35 ^{c}	15.8 ± 0.84 ^{b}	178.4 ± 12.78 ^{c}	3	22.9 ± 0.70 ^{c}	6 August	6 November
IT273590	133.2 ± 9.83 ^{d}	9.4 ± 2.70 ^{b}	22.0 ± 1.22 ^{c}	122.0 ± 38.94 ^{b}	2	15.1 ± 0.61 ^{b}	18 August	15 November

† The different letters indicate a significant difference at $p < 0.05$ by ANOVA and Duncan's test.

4. Discussion

Soybean TIs interfere with protein digestion in animals and reduce food availability [46]. Although the TIA of soybeans can be decreased by various processing methods, such as heat treatment, starch treatment, electromagnetic wave treatment, and cryogenic treatment, these treatments are expensive and may induce damage to other proteins [47–49]. Therefore, the most effective way to inactivate this nutritional element in soybean meals is to develop KTi-free soybean cultivars. Currently, three soybean accessions (PI157440, PI196168, and PI68679) lacking the KTi have been identified among the USDA soybean germplasm collection and extensively utilized in soybean breeding programs to introduce the null allele of the TI [26,50,51]. In this study, we screened many genotypes in Korean soybean germplasm collections to identify new soybean genotypes with low KTi contents and characterize novel genetic variations in KTi genes.

In this study, TIA analysis was conducted on a large number of soybean accessions using a colorimetric enzyme assay that relies on enzyme–substrate reactions [37]. However, enzyme–substrate reactions are significantly influenced by temperature and incubation time. Lowering the temperature of the reagent or reducing the reaction time may lower the TIA content. In this experiment, to maintain a consistent temperature, the assay buffer was pre-stored in a dry bath at 40 °C to create the BAPA working solution. Additionally, the protein extract was refrigerated at 2 °C. As the temperature of the protein extract approached room temperature, lower values were observed in soybean varieties with low KTI content. However, there was no significant reduction in varieties with high KTI content. This suggests that the modified assay in this study effectively screens for KTi and allows for the accurate and rapid screening of low TIA levels.

Among the 999 soybean accessions, more than 88% of genotypes exhibited a high level of TIA, ranging from 90% to 100% (Figure 1). Additionally, we identified six soybean accessions with low TIA levels (below 60%) similar to the KTi-free genotypes of PI542044 (Table 2). Among these six genotypes, three accessions (IT274515, IT273590, and IT274513) originated in Korea (Table 2). Interestingly, two KTi-3 null lines, PI157740 and PI196168, were also introduced into the USA from South Korea [52]. To determine if the low TIA levels in these six accessions were associated with KTi-3 null lines, the coding regions of the KTi-3 gene (*Glyma.08g341500*) were compared with PI542044. A KASP analysis revealed that IT274513, IT274515, IT276197, and IT022891 carried the same mutation as PI542044. Since these three accessions were recently deposited at the RDA GenBank in 2013–2014, the low KTi trait in IT274513, IT274515, and IT276197 might have originated from breeding programs utilizing these KTi-3 null lines.

Re-sequencing analysis is the most effective method for identifying genetic variations in candidate genes. Jofuku and Goldberg reported a minimum of 10 KTi genes in the soybean genome. [20]. In this study, KTi genes were identified from the Williams82 soybean reference genome (Wm82.a2.v1.genome). Using re-sequencing analysis, we examined the genetic variations of the gene in three soybean accessions (IT105782, IT170889, and IT273590) containing no KTi-3 gene mutation. Remarkably, we found that IT105782 contained a three-base-insertion mutation (A461 → ACTA) and five missense mutations in the coding region of the KTi-1 gene (*Glyma.01g09500*) (Figure 3). The insertion of an additional three-base pairs led to the insertion of one more amino acid (Y154 → YY154), without resulting in a frameshift change. The previously reported KTi-1 null line, PI68679, had a single-base deletion (GGG → 358GG) at position 356 bp, resulting in a frame shift and a truncated protein [21]. Therefore, the mutation found in IT105782 differed from PI69679, suggesting that it is a new mutant allele of the KTi-1 gene.

To evaluate the impact of the novel KTi-1 gene mutation on mRNA levels, we conducted RT-qPCR analysis on mature seeds. The results showed a significant reduction (17–63%) in the expression level of the KTi-1 gene in IT105782 compared to the wild type Daepung 2 during the mid-maturation and late-maturation stages (Figure 4). This reduction suggests a direct correlation between the reduced KTi-1 mRNA level and the decline in TI protein production. Moreover, it is crucial to consider the suitability of these three soybean varieties for cultivation. An assessment of various agricultural characteristics, including average seed weight, seed count, and other quantitative aspects, indicated that these varieties may not be ideal choices for cultivation. In the development of genetic markers related to KTi-1, some efforts have faced challenges and been unsuccessful [21]. Despite these challenges, we successfully developed the KTi-1 KASP marker, demonstrating its effectiveness in distinguishing between the mutation type and the wild type.

5. Conclusions

In this study, we identified several soybean accessions with low TIA from a pool of 999 soybean germplasm accessions. Among them, IT105782 had an in-frame insertion (A461 → ACTA) mutation and five missense mutations in the *Glyma01g095000* gene. This mutation is a novel KTi-1 gene mutation that has not been previously reported. RT-qPCR analysis revealed that the KTi-1 mRNA expression level in IT105782 significantly decreased by 17%-63% compared to Daepung 2 (an elite soybean variety) during the two seed stages. IT105782 showed early maturation characteristics similar to those of null-KTi soybean, although its other agronomic traits were not superior to those of Daepung 2 (an elite cultivar soybean). This study identified a new KTi-1 mutation and developed a new KTi-1 molecular marker. Breeder-friendly KASP markers for selecting KTi-null plants will soon be readily available in numerous breeding programs.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture13112070/s1. Figure S1: Base sequence of IT105782 in KTi-1 *Glyma01g095000* gene. Sequence variations are shown in square brackets, with the front-base sequence representing reference and the back-base sequence representing IT105782; Figure S2: Amino

acid differences between IT105782 and reference in KTi-1 *Glyma01g095000* gene; Table S1: Trypsin inhibitor activity (TIA) in 999 soybean accessions.

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