Development of Breeder-Friendly KASP Markers for Low Concentration of Kunitz Trypsin Inhibitor in Soybean Seeds

M. Luciana Rosso 1, Chao Shang 1, Qijian Song 2, Diana Escamilla 3, Jay Gillenwater 4 and Bo Zhang 1,*

1 School of Plant and Environmental Sciences, Virginia Tech, Blacksburg, VA 24061, USA; luciana@vt.edu (M.L.R.); chshang@vt.edu (C.S.)
2 Beltsville Agricultural Research Center, Soybean Genomics and Improvement Laboratory, USDA-ARS, Beltsville, MD 20705, USA; qijian.song@ars.usda.gov
3 Department of Agronomy, Purdue University, West Lafayette, IN 47907, USA;descamil@purdue.edu
4 Department of Crop and Soil Sciences, North Carolina State University, Raleigh, NC 27695, USA; jhgille2@ncsu.edu
* Correspondence: bozhang@vt.edu

Abstract: Trypsin inhibitors (TI), a common anti-nutritional factor in soybean, prevent animals’ protein digestibility reducing animal growth performance. No commercial soybean cultivars with low or null concentration of TI are available. The availability of a high throughput genotyping assay will be beneficial to incorporate the low TI trait into elite breeding lines. The aim of this study is to develop and validate a breeder friendly Kompetitive Allele Specific PCR (KASP) assay linked to low Kunitz trypsin inhibitor (KTI) in soybean seeds. A total of 200 F3:5 lines derived from PI 547656 (low KTI) X Glenn (normal KTI) were genotyped using the BARCSoySNP6K_v2 Beadchip. F3:4 and F3:5 lines were grown in Blacksburg and Orange, Virginia in three years, respectively, and were measured for KTI content using a quantitative HPLC method. We identified three SNP markers tightly linked to the major QTL associated to low KTI in the mapping population. Based on these SNPs, we developed and validated the KASP assays in a set of 93 diverse germplasm accessions. The marker Gm08_44814503 has 86% selection efficiency for the accessions with low KTI and could be used in marker assisted breeding to facilitate the incorporation of low KTI content in soybean seeds.

Keywords: Kunitz trypsin inhibitor; soybean; quantitative trait loci; anti-nutritional factor; single nucleotide polymorphism; KASP

1. Introduction

Soybean (Glycine max L. Merr.) is widely recognized as the richest and least expensive source of vegetable protein for livestock, poultry and aquaculture production, which is also used as a source of metabolizable energy (http://soystats.com, date: December 18, 2020). However, soybean seeds have several anti-nutritional factors (ANFs) that affect animal nutrient digestion and absorption, reducing animal growth performance in meat production [1]. The main ANFs in the soybean seeds include proteinase inhibitors, metal chelates, oligosaccharides and antigenic factors. Although the meal industry has established solutions, such as roasting that is applied on raw soybean meal to inactive ANFs, heating can degrade certain essential amino acids, and the feed cost becomes higher due to higher energy cost [2]. Therefore, the most economical and reliable way to improve animals’ ability to digest protein is to feed them with soybean meals containing low concentration of ANFs.

Trypsin inhibitor (TI), one of the ANFs in soybeans, is the major proteinase inhibitor accounting for about 6% of the total protein in soybean seed [3]. TI restrains the activity of a protein-digesting enzyme called trypsin, causing pancreatic hypertrophy/hyperplasia, which ultimately results in an inhibition of animal growth [4]. Trypsin inhibitor in
soybean seeds includes two class families, Kunitz TI (KTI, 21 KDa) which is sensitive to thermal treatment and has specificity for trypsin, and Bowman–Birk TI (BBTI, 7–8 KDa), which is more thermo-stable, capable to inhibit trypsin and chymotrypsin [5,6]. Among these two types of TI, KTI serves as the major contributor to the trypsin inhibitor activity in soybeans. KTI is encoded by a gene family containing more than 10 independent genes [7]. Only two KTI genes (KTI3 and GMKTI-1) are sequenced and mapped on chromosome 8 (NCBI.gov, accessed in April 13 2020). KTI3 is the major contributor of the KTI phenotype and is expressed in the soybean seeds [8]. Wang et al. (2011) reported 13 iso-forms of KTI, which are governed by a single gene with multiple alleles [9]. BBTI family in soybean encompass many genes having smaller impacts on the TI phenotype.

Two accessions were originally identified from the USDA’s Germplasm collection: PI 157,440 (‘Kin-du’) and PI 196168, completely lacking the Kunitz trypsin inhibitor protein [10] and both were shown to have a frameshift mutation in the KTI3 gene (kti) [7,11]. Despite this mutation, a wide range in trypsin inhibitor activity is observed among germplasm containing either wild type KTI3 or null kti3 allele [12,13] due to a significant proteome rebalancing in KTI mutant lines, resulting in an increased BBTI protein levels [14]. Additionally, both Kunitz and Bowman–Birk trypsin inhibitor classes are influenced by the environment, creating a challenge in breeding selections [12,13,15]. The breeding effort of low TI or TI-free activity seemed very slow [16,17] after ‘Kunitz’ variety, with PI 157,440 as the KTI free donor, was released in the 1970’s, probably because of the establishment of commercial soybean meal processing. Additionally, soybean breeders’ efforts have been focusing on the improvement of productivity characteristics of soybeans such as yield, resistance to disease as well as seed composition such as high protein and high oil concentration. Breeding soybeans for low trypsin inhibitors could potentially replace heat treatment to remove trypsin inhibitor and overcome the problem of reducing nutritional value of soybean meal after heating.

The use of molecular markers for marker-assisted selection (MAS) have been proved to speed up the breeding process and efficiently introgress recessive alleles into crops [18–20]. Up to date, previous studies on the development of low KTI soybean cultivars reported the use of SSR markers as tool for breeding selection [21–25]. SSR markers in Chr 08 such as SSR 228, SSR 409 and SSR 429 were reported to be closely linked (0–10 cM) to ktti, the recessive form of KTi gene [24]. However, SSRs are generally abundant and polymorphic in non-expressed genomic regions and consequently considered to be selectively neutral. In recent years, SNP markers have started to replace SSRs in population genetic studies as well as in a wide range of other applications [26,27]. SNPs occur in genomes at a much higher frequency than SSRs, but they also occur in intergenic and non-coding regions. However, genome-wide association studies revealed that SNPs located in non-coding regions are often physically linked to functional or regulatory genomic sites, thus reflecting, for example, selection signatures [28]. Moreover, SNP markers are highly stable and can be genotyped in high-throughput systems with a high multiplex ratio such as the PCR-based Competitive Allele Specific technology (KASPar) that is simpler and more cost-effective than other SNP assays.

In a recent article, Patil et al. (2017) reported a development of a SNP genotyping assay for detection of low KTI in soybean in an F2 population of SP6A-206 x PI 542,044 (low KTI). However, Patil et al. did not report what phenotyping system they used to discriminate the low KTI lines from the high ones, making not clear if the KASP clustering correspond to true low and high KTI phenotypes. Moreover, we tested the reported SNP genotyping assay in the Glenn (high KTI) x PI 547,656 (low KTI) population with no successful results, perhaps because PI 542,044 and PI 547,656 do not have common low KTI genes. In 2018, Rosso et al. developed an HPLC method as a reliable method to quantify KTI in soybean seeds. showing that this quantification method was strongly correlated with two popular activity assays for measuring total TI activity. Additionally, the HPLC method overcomes the flaws of activity assays because it can detect low levels of KTI on nonfunctional mutated soybean lines, which is impossible for activity assays, and can
measure only KTI, not the mixture of KTI and BBTI as activity assays do. Therefore, the objectives of this study were: (i) to identify SNP markers associated with the QTL that confers low KTI in soybean seeds; and (ii) to develop and validate a breeder-friendly KASP SNP genotyping assay linked to low KTI content derived from ‘Kin-du’ as the KTI free donor, that could be used in marker-assisted selection to incorporate the low KTI trait into elite breeding lines.

2. Results

2.1. Kunitz Trypsin Inhibitor Phenotyping

The HPLC method successfully quantified the concentration of KTI in soybean seeds from the two selected parents, F3:4 (data not shown) and F3:5 lines from the mapping population and the 93 PIs selected for the SNP validation. Analysis of variance and least squares means Student’s $t$-test showed there was a significant ($p = 0.007$) variation in KTI concentration between the two parents, Glenn and PI 547,656 (Tables 1 and 2). There was no significant difference between location and the parent by location interaction was not significant either (Tables 1 and 2). Analysis of variance indicated that there was a significant difference among the F3:5 lines (Table 3) on KTI concentration, ranging from 0.05 mg/g to 24.14 mg/g (standard errors = 0.12) (Figure 1). The non-parametric Kruskal–Wallis test and analysis of variance showed that there was a statistically significance difference in KTI concentration between the different locations, Blacksburg and Orange ($x^2 = 3.78$, $p = 0.05$) and also between years ($x^2 = 164.76$, $p < 0.0001$) (Tables 3 and 4). Frequency distribution of the 93 PIs selected for SNP validation also showed a wide range of KTI concentration, ranging from 0.8 mg/g to 8.02 mg/g (Figure 2).

The broad-sense heritability ($H^2$) of KTI was estimated to be 54% and was calculated using variance components for KTI of the mapping population across two years (2016 and 2017) and two locations (Blacksburg and Orange, VA, USA).

Table 1. Analysis of variance of Kunitz trypsin inhibitor concentration (KTI) between the two parents Glenn (normal KTI) and PI 547,656 (low KTI) planted in Blacksburg and Orange, VA in 2016 and 2017.

| Effect                   | df  | Sum of Squares | $f$-value | p-value |
|--------------------------|-----|----------------|-----------|---------|
| Environments             | 3   | 63.36          | 1.33      | 0.27    |
| Residual Error           | 14  | 1131.39        |           |         |
| Parent                   | 1   | 505.80         | 10.50     | 0.017   |
| Residual Error           | 14  | 688.95         |           |         |
| Parent by Location       | 3   | 50.83          | 10.60     | 0.32    |
| interaction              |     |                |           |         |
Table 2. Least squares mean differences using Student’s t test of Kunitz trypsin inhibitor concentration (KTI) between the two parents Glenn (normal KTI) and PI 547,656 (low KTI) planted in Blacksburg and Orange, VA in 2016 and 2017.

| Level       | Student’s t test Grouping | Least Square Mean | α  | t-value |
|-------------|---------------------------|-------------------|----|---------|
| Glenn       | A                         | 12.63             | 0.05 | 2.18    |
| PI 547656   | B                         | 1.38              |     |         |
| Blacksburg  | A                         | 8.34              | 0.05 | 1.96    |
| Orange      | A                         | 8.06              |     |         |

Levels not connected by same letter are significantly different. * level of significance α = 0.05.

Table 3. Analysis of variance of Kunitz trypsin inhibitor concentration (KTI) among 200 F3:4 lines (2016) and 200 F3:5 lines (2017) as well as parents Glenn (normal KTI) and PI 547,656 (low KTI) planted in Blacksburg and Orange, VA.

| Effect                  | df  | Sum of Squares | f-value | p-value |
|-------------------------|-----|----------------|---------|---------|
| Genotype                | 201 | 18446.89       | 14.81   | <0.0001 |
| Residual Error          | 1614| 12333.51       |         |         |
| Year                    | 1   | 3644.435       | 588.13  | <0.0001 |
| Residual Error          | 1614| 27135.97       | 10.84   | 0.0001  |
| Location                | 1   | 67.21          | 10.84   | 0.0001  |
| Residual Error          | 1614| 30713.18       | 2.08    | 0.15    |
| Replication             | 1   | 12.86          | 2.08    | 0.15    |
| Genotype by Location interaction | 201 | 1111.05       | 0.89    | 0.82    |

Table 4. Kruskal–Wallis test of Kunitz trypsin inhibitor concentration (KTI) among 200 F3:4 lines (2016) planted in Blacksburg and Orange, VA in 2016 and 2017.

| Level       | Count | Score Sum  | Expected Score | Score Mean |
|-------------|-------|-----------|----------------|------------|
| Location    |       |           |                |            |
| Blacksburg  | 800   | 622434    | 640400         | 778.04     |
| Orange      | 800   | 658366    | 640400         | 822.95     |

1-way Test

| Chi-square | df  | Prob > ChiSq |
|------------|-----|--------------|
| 3.78       | 1   | 0.05         |

| Year       |       |              |                |            |
|------------|-------|--------------|----------------|------------|
| 2016       | 800   | 521809       | 640400         | 652.26     |
| 2017       | 800   | 758991       | 640400         | 948.73     |

1-way Test

| Chi-square | df  | Prob > ChiSq |
|------------|-----|--------------|
| 164.76     | 1   | <0.0001      |

level of significance α = 0.05.
Figure 1. Frequency distribution of KTI concentration (mg/g) of in 200 F$_{3.5}$ lines developed from a cross between Glenn (normal KTI) and PI 547,656 (low KTI) planted in Blacksburg and Orange, VA in 2016 and 2017.

Figure 2. Frequency distribution of KTI concentration (mg/g) of Plant Introductions (PIs) selected for the SNP Validation.
2.2. QTL Analysis

Out of 6000 evaluated SNPs, a total of 1357 polymorphic SNPs (22.6%) were selected for establishing the genetic linkage map. The linkage map generated by SMA from the mapping population consisted of 20 chromosomes, which spanned 1278.69 cM and were defined by 1272 SNP markers. The constructed map had a coverage ranging from 0.097 to 0.312 cM with an average coverage of 0.215 cM (Table 5). One QTL conferring low KTI concentration was identified using IciMapping analysis. The QTL located in chromosome 8 (LG A2) was mapped between SNPs Gm08_44814503 and Gm08_45270892, with a peak close to Gm08_44814503 (Figure 3). QTL detected in 2016 and 2017 showed LOD score of 21.93 and 23.44 and explained 39.95 and 39.83% of the total variation of the trait, respectively (Table 6). This QTL is stable across years and significantly associated with quantities of KTI. We tentatively named this QTL for KTI concentrations as \( q_{\text{KTI08}} \). The additive effect (Add) of \( q_{\text{KTI08}} \) was \(-1.88 \) mg/g in 2016 and \(-3.99 \) mg/g in 2017, indicating that the allele at this QTL inherited from the low KTI parent (PI 547656) reduced KTI content (Table 6). When analyzing the average KTI content across 2016 and 2017, \( q_{\text{KTI08}} \) showed a LOD score of 24.95, explained 41.34% of total phenotypic variation, and had a \(-2.93 \) mg/g additive affect (Table 6).

Table 5. Summary of single nucleotide polymorphism (SNP) marker mapping in F3-derived F5 mapping population from Glenn x PI 547656.

| Chr | LG | Length (cM) | No. all SNPs | All SNP coverage | No. P-SNPs | P-SNPs coverage | No. of mapped SNPs |
|-----|----|-------------|--------------|-----------------|------------|-----------------|-------------------|
| 1   | D1a| 70.90       | 263          | 0.270           | 71         | 0.999           | 71                |
| 2   | D1b| 88.42       | 329          | 0.269           | 80         | 1.105           | 79                |
| 3   | N  | 50.14       | 280          | 0.179           | 86         | 0.583           | 72                |
| 4   | C1 | 38.42       | 266          | 0.144           | 64         | 0.600           | 50                |
| 5   | A1 | 87.63       | 294          | 0.298           | 95         | 0.922           | 95                |
| 6   | C2 | 95.03       | 313          | 0.304           | 86         | 1.105           | 86                |
| 7   | M  | 55.13       | 319          | 0.173           | 45         | 1.225           | 45                |
| 8   | A2 | 92.40       | 380          | 0.243           | 91         | 1.015           | 90                |
| 9   | K  | 81.39       | 261          | 0.312           | 77         | 1.057           | 75                |
| 10  | O  | 66.34       | 317          | 0.209           | 77         | 0.862           | 72                |
| 11  | B1 | 63.03       | 279          | 0.226           | 39         | 1.616           | 39                |
| 12  | H  | 68.79       | 258          | 0.267           | 45         | 1.529           | 44                |
| 13  | F  | 70.78       | 381          | 0.186           | 61         | 1.160           | 60                |
| 14  | B2 | 27.58       | 275          | 0.100           | 66         | 0.418           | 38                |
| 15  | E  | 65.48       | 305          | 0.215           | 71         | 0.922           | 70                |
| 16  | J  | 45.66       | 238          | 0.192           | 54         | 0.846           | 54                |
| 17  | D2 | 55.85       | 265          | 0.211           | 41         | 1.362           | 40                |
| 18  | G  | 37.01       | 380          | 0.097           | 107        | 0.346           | 92                |
| 19  | L  | 40.36       | 326          | 0.124           | 41         | 0.984           | 40                |
| 20  | I  | 78.35       | 271          | 0.289           | 60         | 1.306           | 60                |

Mean 63.93 300 0.215 67.85 0.998 63.60
Total 1278.69 6000 1357 1272

a Chromosome. b Linkage group. c Chromosome length in centimorgan. d SNP, single nucleotide polymorphism. Number of markers screened for each chromosome: all SNPs (all 6K SNPs), and P-SNPs (polymorphic SNPs). e Distribution of SNP markers on chromosomes (total chromosome length/number of SNP markers screened): all SNPs (all 6K SNPs), and P-SNPs (polymorphic SNPs).
Figure 3. Quantitative trait loci (QTL) mapping of \textit{qKTI08} in chromosome 8 (LG A2) in the biparental mapping population derived from Glenn x PI 547656.
Table 6. Quantitative trait loci (QTL) mapping, marker intervals, logarithm of odds (LOD) scores, percentage of variance (PVE), and additive effects of QTL for KTI concentration (qKTI08) in the F3:5 mapping population derived from Glenn x PI 547656.

| Year | QTL Name | Chr. (LG) a | Position (cM) | Confidence Interval b | Peak | LODc | PVE (%) d | Add (mg/g) e |
|------|----------|-------------|---------------|-----------------------|------|------|-----------|-------------|
| 2016 | qKTI08 8 (A2) | 86 | Gm08_44814503 Gm08_4481450 | 3 | 21.93 | 39.95 | -1.88 |
| 2017 | qKTI08 8 (A2) | 85 | Gm08_44814503 Gm08_4481450 | 3 | 23.44 | 39.83 | -3.99 |
| Avg 2016–2017 | qKTI08 8 (A2) | 86 | Gm08_44814503 Gm08_4481450 | 3 | 24.95 | 41.94 | -2.93 |

a Chromosome (Linkage Group). b Physical position of interval markers in base pairs on the William 82 reference genome (Wm82.a2.v1). c Logarithm of odds. d Percentage of variance explained. e Additivity effect (mg/g) on the specific QTL (a negative effect indicated a low concentration of KTI contributed by PI 547656).

The SNPs Gm08_44814503 and Gm08_45270892 were located at 85.22 and 88.74 cM on chromosome 8 (LG A2), respectively, in the most recently developed high density linkage map for soybeans [29]. Based on the physical position information of the SNPs, the physical distance between these markers is 1.91 Mb; and the corresponding genome positions of Gm08_44814503 and Gm08_45270892 in the reference soybean genome (Glyma.Wm82.a2.V1 assembly) are 35.14 Mb–37.05 Mb [29].

2.3. SNP Validation

Molecular markers flanking the qKTI08 region on chromosome 8 that were significantly associated (p-value < 0.0001) with low Kunitz trypsin inhibitor content across years and locations were identified using single marker analysis (SMA) on IciMapping. Table 7 shows five SNPs significantly associated with KTI content in the mapping population in 2016 and 2017. All these SNPs are tightly linked to qKTI08 (Figure 2). We converted three of them to KASP assays to validate the robustness of these SNP assays on selected 93 plant introductions with low and high KTI content. Out of the 93 PIs phenotyped for KIT concentration by HPLC analysis, 51 lines were normal KTI and 42 were low KTI. Table 2 shows the selection efficiency of these three SNP markers. The three SNP markers, Gm08_44265646, Gm08_44814503 and Gm08_45317135, were polymorphic between the PIs and have a selection efficiency of 64%, 86% and 31%, respectively. Out of a total of 42 PIs with low KTI phenotype, 15 (36%) and 29 (69%) low KTI PIs were not selected by Gm08_44265646 and Gm08_45317135, respectively. However, only six (14%) low KTI PIs were not selected by Gm08_44814503 SNP marker, which means that Gm08_44814503 SNP marker was able to select 86% of the low KTI PIs from the core collection (Table 2). Figure 4 shows the results of the cluster analysis of Gm08_44814503 SNP marker where 31 lines showed the normal KTI genotype, three lines showed the heterozygous genotype and 59 lines showed the low KTI genotype, the last two samples in the graph belong to Glenn (normal KTI) and PI 547,656 (low KTI).
Figure 4. KlusterCaller™ genotyping results. Gm08_44814503 SNP genotyping results of the 93 PIs and Glenn (well 12F) and PI 547,656 (well 12G) are shown in the Cartesian cluster plot. Blue data points are homozygous lines for the low KTI allele reported by FAM dye, green data points are heterozygous lines and red data points are homozygous lines for the normal KTI allele reported as HEX dye. The black data point represents the no template control (NTC). The DNA sample 96-well plate layout is shown below the cluster plot.

Table 7. KASP assay primers sequences significantly associated ($p < 0.0001$) with concentration of KTI in the mapping population derived from Glenn x PI 547656.

| Chr. (LG) a | Marker ID | Position (cM) | Sequences | Low KTI Allele b | Will8 2 Allele c | LOD d |
|-------------|-----------|---------------|-----------|-----------------|-----------------|-------|
| 8 (A2)      | Gm08_44265646_C _T | 84.42 | FAM_primer: GAAGGTGACCAAGTTCTATGCTTTATGCAC-GCCTGAAACTTTA<br>VIC_primer: GAAGTCCGAGTCACGATTCTATGAC-GCATGGAATTGTTG<br>Common reverse primer: TGTACTCACCTTGGAAACTTCT | T | C | 21.68 *** |
| 8 (A2)      | Gm08_44814503_C _T | 85.22 | FAM_primer: GAAGGTGACCAAGTTCTATGCTTTATGCAC-GCCTGAAACTTTA<br>VIC_primer: GAAGTCCGAGTCACGATTCTATGAC-GCATGGAATTGTTG<br>Common reverse primer: TGTACTCACCTTGGAAACTTCT | T | C | 21.73 *** |
3. Discussion

The presence of Kunitz trypsin inhibitor (KTI) in soybean seeds has a negative impact on the feed and food industry since it affects animal nutrient digestion and absorption, reducing animal growth performance in meat production [4]. The best cost-efficient way to overcome this problem would be to use soybean cultivars with low or null KTI content. In an effort to develop soybean cultivars with low KTI content, we crossed the soybean cultivar Glenn (normal KTI content of 12.63 mg/g) with the low KTI PI 547,656 (1.38 mg/g) that carries the low KTI allele in 2014. The biparental population of 200 F3:5 lines presented a wide range of KTI content (0.5–24.14 mg/g) (Figure 5). We estimated that $H^2$ for KTI content as 0.54, which is considered a medium heritability [30]. A moderate broad-sense heritability and significant difference of KTI content between years and locations (Tables 3 and 4) showed that genetic and environment affect this trait. Vollmann et al. (2003) also reported that KTI activity in soybean was significantly affected by the environment and genotype. Thus, the use of molecular markers would play an important role to incorporate the low KTI trait into elite breeding lines. Molecular marker-based selection paired with phenotypic selection is an efficient way to advance breeding lines with the desirable trait, plus selective elimination of undesirable traits, in the shortest possible time. Microsatellite markers, such as Satt 228, Satt 409 and Satt 429 have been previously used when breeding for low or null KTI content [24,31,32], however, single nucleotide polymorphism markers have gained importance in the breeding programs due to their large amount on the genome, high cost efficiency, and high repeatability.

This study confirms the presence of a QTL on Chromosome 8 (LG A2) associated with low concentration of KTI previously reported. In this study, one QTL ($qKTI08$) associated with KTI was detected in PI 547,656 on chromosome 8 with a confidence interval from Gm08: 44,814,503 to Gm08:45270892 (Figure 2). This QTL is desired for marker-assisted selection due to its stability across years and locations. As today, there are five published genes in this region, two calcineurin B-like protein, two heat shock cognate protein and one proliferating cell nuclear antigen [33]. However, close to this region, between Gm08_45730511 and Gm08_45786029 SNP markers, seven Kunitz trypsin inhibitor proteins have been identified, including KTI3 [34,35]. Therefore, chromosome 8 harbors several genes associated with Kunitz trypsin inhibitors. Moreover, previously published SSR markers used for breeding low TI lines [24,31,32], however, single nucleotide polymorphism markers have gained importance in the breeding programs due to their large amount on the genome, high cost efficiency, and high repeatability.

We found five SNP markers closely associated ($p < 0.0001$) with low KTI content across years and locations (Table 7). Three out of these five SNP markers were converted to KASP assays and use for validation on the genetically diverse 93 PIs. Gm08_44814503
KASP SNP assay was able to select 86% of the low KTI PIs from the validation panel. This SNP marker will be more useful in MAS than previously developed assay by Patil et al., 2017, since our was validated in 93 plant introductions that have much more diverse genetic background than Kin-du alone as the low KTI donor. The SNP assay previously developed by Patil et al., 2017 for low KTI was based on a mutation on PI 157,740 [7], however they only tested this assay on an F2 population derived from SP6A-209 x PI 542,044 lines, where PI 542,044 is a germplasm with null KTI. The validated KASP assay proposed in this work offers a rapid and more economical genotyping assay compared to previous assays [36,37] in practical applications such as MAS backcrossing, advance breeding lines, and germplasm identification. Moreover, KASP assays has been proven to work very well for many traits in the soybean genome [38].

Current published TI activity assays can only test total TI activity, but cannot differentiate KTI or BBTI activity. Therefore, we focused on KTI content in this study. However, breeding only for low or null KTI content in soybean would not be sufficient for practical applications for a few reasons, such as the effect of environmental conditions on the trait, the interaction of environment and genotype, the expression of one of the 13 iso-forms of KTI3 gene in the seeds and presence of Bowman–Birk TI affect the TI activity in soybean seeds. Gillman et al. indicated that low KTI lines had dramatically increased Bowman–Birk TI level [14]. Therefore, the practical and effective approach for expanding the soybean meal value for the poultry industry is to reduce both KTI and BBTI content in soybean. The implementation of low total TI into breeding lines would reduce the need of postharvest heat treatment, which will bring benefits to the soybean feed and food processing industry.

In conclusion, this study successfully mapped the QTL on chromosome 8 linked to KTI content that was previously published and developed a robust, cost-effective and high-throughput SNP assay that could be applied across different genetic backgrounds in soybean breeding programs to facilitate marker-assisted selection for low KTI content in soybean seeds with great efficiency and accuracy. The KASPpar KTI assay is a breeder-friendly SNP detection system for selection of low KTI soybean lines since KASPpar assay samples are amplified with a standard thermal cycler and can be genotyped with any type of FRET reader, which can easily be done by many breeding programs. Future work will focus on the identification of QTLs and SNP markers to select soybean breeding lines low in BBTI content as well, as double null will have a stronger application to the feed and food industry.

4. Methods

4.1. Population Development

Parental soybean lines were chosen based on the amount of KTI in the seeds. Glenn, developed and released by Virginia Tech in 2009, was chosen as a normal KTI concentration parent. PI 547,656 (cultivar name: L81-4871) was chosen as a low KTI parent. PI 547656, derived from a cross ‘Clark 63’ x PI 157,440 (‘Kin-du’), was developed by the USDA-ARS and the Illinois Agricultural Experimental Station in 1986 [15]. Glenn X PI 547,656 crosses were made in summer of 2014 at the Virginia Tech farm in Blacksburg, VA. F1 plants were spaced-planted and increased for two generations with modified pod descent at each generation in a Puerto Rico winter nursery in winter of 2014. Six SSR markers (Satt449, Satt197, Satt281, Satt268, Satt431 and Satt345), which were polymorphic between parents, were used to verify true hybrids. A total of 200 individuals of the F2 were advanced to F3 in 2015. The parents and F23 lines were separately planted in single, 3.05 m long rows with 0.76 m row spacing and about 30 seeds per meter arranged in a randomized complete block design in Blacksburg, VA, USA in 2015. Two parents and a population of F34 lines were grown in Blacksburg and Orange, VA, USA in 2016, and two parents and a population of F35 lines were grown in the same two locations in 2017 using the same experimental design.
4.2. Kunitz Trypsin Inhibitor Phenotyping

The two parents and 200 progenies of the F3:4 and F3:5 mapping population were phenotyped for KTI content using a quantitative HPLC method [39]. F3:4 phenotypic data are not shown. Briefly, 10 mg of finely ground soybean seed powder was mixed with 1.5 mL of 0.1 M sodium acetate buffer, pH 4.5. Samples were vortexed and shaken for 1 h at room temperature. The samples were centrifuged at 12,000 rpm for 15 min. One-mL of the supernatants were filtered through a syringe with an IC Millex-LG 13 mm mounted 0.2 µm low protein binding hydrophilic millipore (PTFE) membrane filter (Millipore Ireland BV, Carrigtwohill, Republic of Ireland). The Kunitz trypsin inhibitor in solution were separated on an HPLC Agilent 1260 Infinity series (Agilent Technologies, Santa Clara, CA, USA), equipped with a guard column (4.6 × 5 mm²) packed with POROS® R2 10 µm Self Pack® Media and Poros R2/H perfusion analytical column (2.1 × 100 mm², 10 µm). The mobile phase A consisted of 0.01% (v/v) trifluoroacetic acid in Milli-Q water and mobile phase B was 0.085% (v/v) trifluoroacetic acid in acetonitrile. The injection volume was 10 µL and the detection wavelength was 220 nm. The final KTI concentration of each sample was calculated using the initial weight of each sample to calculate the KTI concentration on a dry weight basis with results reported as milligrams per gram (mg/g).

4.3. DNA Extraction

A bulk sample of young leaves from three individuals of each F3:5 lines of the mapping population and each PI from the validation panel were collected and stored at −80 °C until DNA extraction. Leaves were freeze dried using a FreeZone 6 Liter Console Freeze Dry System (−56 °C and 0.220 mbar) and about 200 mg of leaf tissue were placed in a 2.5 mL tube. Tissue was ground to fine powder in liquid N2 using glass stirring rods. Total genomic DNA was isolated from leaf tissue (0.20 g) by a modified protocol from the CTAB method of [40].

4.4. Single-Nucleotide Polymorphism Genotyping

Fifty nanograms of genomic DNA for the parental lines and the population of F3:5 lines were sent to USDA–ARS Soybean Genomics and Improvement Laboratory (Beltsville, MD, USA) and genotyped using the Illumina BARCSoySNP6K_v2 Beadchip containing 6000 SNPs. Single-nucleotide polymorphism genotyping was conducted according to Song et al. 2013 [41] on the Illumina platform following the Infinium HD Assay Ultra Protocol (Illumina, Inc.). Single-nucleotide polymorphism allele calling was done using the Genome Studio Module v2.0.3 software (Illumina, Inc.). The low KTI parent was scored as A and high KTI parent was scored as B. SNPs with no call and the monomorphic SNPs between parents were discarded. SNPs with low minor allele frequency (MAF) (<10%) and high missing data ratio (<5%), as well as severe segregation distortion were filtered for quality control.

4.5. Linkage Map Construction and QTL Analysis

Linkage maps were constructed by JoinMap v4 [42] using a regression approach with a minimum logarithm of odds (LOD) threshold of 3 for linkage group construction. Recombination frequencies were converted to centimorgan (cM) using Kosambi’s mapping function [43]. QTL analysis was performed by single marker analysis, interval mapping and composite interval mapping implemented in IciMapping v 4.1 [44]. For single marker analysis, SMA, \( p < 0.0001 \) was used as a threshold for significant markers. In the composite interval mapping (CIM) and simple interval mapping (SIM), the empirical significance threshold was determined by 1000-time permutation with a walk speed of 1 cM and significance level of 0.05. MapChart [45] was used to create the LOD plots based on JoinMap v4 and IciMapping v4.1 data.
4.6. KASP Marker Development

The SNPs tightly linked to major QTL identified in the mapping population (Table 7) were converted into Kompetitive Allele Specific PCR (KASP) SNP genotyping assays (LGC, Middlesex, UK) with the flanking sequences obtained from the *G. max* genome Glyma.Wm82.a1 (Schmutz et al. 2010) following Zhu et al. [46]. Briefly, the KASP oligos were synthesized by IDT (IDT, Iowa, USA), with primers carrying FAM tail (5′-GAAGGTGACCAAGTTCATGCT-3′) or VIC tail (5′-GAAGGTCCGAGTCAACCGAT-3′), and the target SNP in the 3′ end. Primer mix and PCR reaction was set up following LGC Genomics recommendation (46 µL distilled water, 30 µL common primer [100 µM], and 12 µL of each tailed primer [100 µM]) [46]. Thermocycling conditions consisted of the initial hot-start step at 95 °C for 15 min., followed by 10 cycles of touchdown PCR (annealing 65 °C to 57 °C, decreasing 0.8 °C per cycle), then 35 cycles of 20 s at 94 °C and 60 s at 57 °C. PCR and fluorescent endpoint reading were performed in FLUOstar Omega microplate reader (BMG LABTECH) [46].

4.7. KASP Marker Validation

The 6749 MGs IV and V soybean accessions collected by the National Genetic Resources Program (USDA Soybean Germplasm Collection) were arbitrarily grouped in 500 clusters based on their genetic distance which were calculated using 42,509 SNPs that were included in the SoySNP50K [47]. One accession with the highest average genetic distance within each cluster was selected to form a collection with 500 accessions. These 500 accessions were planted in single, 1.83 m long rows with 0.15 m row spacing and about 30 seeds per meter arranged in a complete randomized design with no replication in Puerto Rico in the winter of 2016, and in a randomized complete block design with three replications in Blacksburg, VA, USA and Clayton, NC, USA in 2017. The 500 accessions were phenotyped for KTI content by HPLC analysis (Figure 5) and 93 diverse plant introductions (PIs) were selected based on KTI content to have a wide range of KTI concentration for the SNP validation. Out of these 93 PIs, 42 were low KTI and 51 were normal KTI. We selected 93 PIs to fill a 96-well plate for PCR amplification (93 PIs, plus Glenn as a negative control, PI 547,656 as a positive control and one blank well). DNA was extracted from bulk leaves of 6–8 plants of each of the selected 93 diverse PIs for the SNP validation. DNA was extracted following the same procedure as used for the mapping population. Selection efficiency (SE) of the selected KASP markers linked to low KTI was calculated as follows: SE = total number of marker-selected lines with low KTI phenotype + total number of lines with low KTI phenotype x 100 (Table 8).
Figure 5. Frequency distribution of KTI concentration (mg/g) of 500 soybean accessions collected by the National Genetic Resources Program (USDA Soybean Germplasm Collection).

Table 8. Selection efficiency of the SNP assays in 93 Plant Introductions (PIs) collected by the National Genetic Resources Program to form a MGs IV and V core collection.

| Phenotype                  | Number of Accessions (n) | Gm08_44265646 | Gm08_44814503 | Gm08_45317135 |
|----------------------------|--------------------------|---------------|---------------|---------------|
| Normal KTI (mg/g) a         | 51                       | 48            | 31            | 64            |
| Heterozygous               |                          | 0             | 3             | 6             |
| Low KTI (mg/g)             | 42                       | 45            | 59            | 23            |

No. low KTI lines selected by marker

| Phenotype                  | Number of Accessions (n) | Gm08_44265646 | Gm08_44814503 | Gm08_45317135 |
|----------------------------|--------------------------|---------------|---------------|---------------|
| Normal KTI (mg/g) a         | 27                       | 36            | 13            |
| Heterozygous               |                          | 15            | 6             | 29            |

Marker selection Efficiency b

| Genotypic Class (n) | Number of Accessions (n) | Gm08_44265646 | Gm08_44814503 | Gm08_45317135 |
|---------------------|--------------------------|---------------|---------------|---------------|
|                      |                          | 64%           | 86%           | 31%           |

\textsuperscript{a} Normal KTI phenotypes consist of wild type and heterozygous phenotypes. \textsuperscript{b} Marker selection Efficiency: Total number of marker-selected lines with low KTI phenotype ÷ total number of lines with low KTI phenotype × 100.

4.8. Statistical Data Analysis

Concentration of KTI, collected in 2016 and 2017, were analyzed using JMP statistical version Pro 14.2.0 (SAS Institute). KTI concentration was analyzed by analysis of variance and the non-parametric method Kruskal–Wallis test for the 2016 and 2017 data at two locations, Blacksburg and Orange, VA, USA. Student’s t test was used to determine if mean of KTI concentration of parents and lines differed significantly at \( p = 0.05 \). Histograms of KTI concentration were also performed by JMP. Analysis of variance was used to determine phenotypic differences between parents, Glenn and PI 547656. Variance-component heritability estimates were calculated by analyses of variance using R software. Broad-sense heritability of seed coat deficiency was estimated using the equation:

\[
H^2 = \frac{s^2_g}{s^2_e + (s^2_{ge}/e) + (s^2/re)}
\]

where \( H^2 \) is heritability, \( s^2_g \) is genotypic variance, \( s^2_{ge}/e \) is genotype x environment interaction variance, \( s^2_e \) is error variance, \( r \) is the number of replications, and \( e \) is the number of environments [48].

Author Contributions: M.L.R. and B.Z. conceived and designed the experiments and wrote the manuscript. M.L.R. conducted the experiments, performed the phenotypic assay, constructed the genetic map and conducted the QTL analysis. B.Z. conceived and oversaw the development of the population. C.S. assisted in the phenotypic HPLC analysis. Q.S. performed the genotyping using the Illumina 6000 SNP INF-BARCSoySNP6K_v2 Beadchip and provided a list of diverse genotypes. D. assisted in genetic mapping and QTL analysis. J.G. helped grinding and weighing the soybean seed samples and assisted in the Kunitz trypsin inhibitor extraction for HPLC analysis. All authors have read and agreed to the published version of the manuscript.

Funding: This study funded by Virginia Soybean Board (VSB) and Virginia Tech Pratt Summer Internship Research Scholarship.

Institutional Review Board Statement: Not applicable

Informed Consent Statement: Not applicable

Data Availability Statement: Not applicable

Acknowledgments: We acknowledge the National Genetic Resources Program of USDA provided

Conflicts of Interest: The authors declare that there is no conflict of interest.
References

1. Yen, J.T.; Jensen, A.H.; Simon, J. Effect of Dietary Raw Soybean and Soybean Trypsin Inhibitor on Trypsin and Chymotrypsin Activity in the Pancreas and in Small Intestinal Juice of Growing Swine. J. Nutr. 1977, 107, 156–165, doi:10.1093/jn/107.1.156.

2. Boge, E.L.; Boylston, T.D.; A Wilson, L. Effect of cultivar and roasting method on composition of roasted soybeans. J. Sci. Food Agric. 2009, 89, 821–826, doi:10.1002/jsfa.3519.

3. Hymowitz, T. Genetics and Breeding of Soybeans Lacking the Kunitz Trypsin Inhibitor. In Nutritional and Toxicological Significance of Enzyme Inhibitors in Foods, Friedman, M., Ed.; Springer: Boston, MA, US, 1986; pp. 291–298.

4. Liener, I.E. Implications of antinutritional components in soybean foods. Crit. Rev. Food Sci. Nutr. 1994, 34, 31–67, doi:10.1080/10408399409527649.

5. Lim, Y.; Xu, Z.; Zhang, C.; Kong, X.; Hua, Y. Heat-Induced Inactivation Mechanisms of Kunitz Trypsin Inhibitor Mutants and Bowman-Birk Inhibitor in Soybean Pro-cressing. Food Chem. 2014, 154, 108–116.

6. Galão, O.; Carrão-Panizzi, M.; Mandarino, J.; Leite, R.; Claus, T.; Visentainer, J. Kunitz Trypsin Inhibitor and Phytic Acid Levels in Conventional and Genetically Modified Soybean Seeds from Londrina and Ponta Grossa, South Brazil. Acta Sci. Technol. 2014, 36.

7. Jofuku, K.D.; Goldberg, R.B. Kunitz Trypsin Inhibitor Genes Are Differentially Expressed During the Soybean Life Cycle and in Transformed Tobacco Plants. The Plant Cell 1989, 1, 1079–1093.

8. De Moraes, R.M.A.; Soares, T.C.B.; Colombo, L.R.; Salla, M.F.S.; Barros, J.G.D.A.; Piovesan, N.D.; De Barros, E.G.; Moreira, M.A. Assisted Selection by Specific DNA Markers for Genetic Elimination of the Kunitz Trypsin Inhibitor and Lectin in Soybean Seeds. Euphytica 2006, 149, 221–226, doi:10.1007/s10681-005-9069-0.

9. Wang; Ke-Jing; Li, Xi.; Yamashita, T.; Takahata, Y. Single Nucleotide Mutation Leading to an Amino Acid Substitution in Kunitz Trypsin Inhibitor. Mol. Breed. 2009, 18, 243–248, doi:10.1007/s11032-013-9930-0.

10. Howell, P.; Leigh, F.; Bates, R.; Gosman, N.; Trafford, K.; Powell, W.; Smith, A.M.; Greenland, A. Rapid marker-assisted development of advanced recombinant lines from barley starch mutants. Mol. Breed. 2013, 33, 243–248, doi:10.1007/s11032-013-9930-0.

11. Kumar, V.; Rani, A.; Shukla, S.; Jha, P. Development of Kunitz Trypsin inhibitor free vegetable soybean genotypes through marker-assisted selection. Int. J. Veg. Sci. 2020, 2, 1–14, doi:10.1080/19315260.2020.1800886.

12. Rani, A.; Kumar, V.; Shukla, S.; Jha, P.; Tayalkar, T.; Mittal, P. Changes in storage protein composition on genetic removal of Kunitz trypsin inhibitor maintain protein content in soybean (Glycine max). J. Agric. Food Res. 2020, 2, 100065, doi:10.1016/j.jafre.2020.100065.

13. Kumar, V.; Rani, A.; Rawal, R. Deployment of Gene Specific Marker in Develop-ment of Kunitz Trypsin Inhibitor Free Soybean Genotypes. Indian J. Exp. Biol. 2013, 51, 1125–1129.

14. Kim, M.S.; Park, M.J.; Jeong, W.H.; Nam, K.C.; Chung, J.I. SSR marker tightly linked to the Ti locus in Soybean [Glycine max (L.) Merr.]. Euphytica 2006, 152, 361–366, doi:10.1007/s10681-006-9223-3.
Van Ooijen, J.W. Joinmap 4®: Software for the Calculation of Genetic Linkage Maps. In Kosambi, D.D. The Estimation of Map Distances from Recombination Values. Annals of Eugenics. Wageningen, The Netherlands, 2006.

SoySNP50K, a High-Density Genotyping Array for Soybean. Song, Q.; Hyten, D.L.; Jia, G.; Quigley, C.V.; Fickus, E.W.; Nelson, R.L.; Cregan, P.B. Development and Evaluation of

Saghai-Maroof, M.A.; Soliman, K.M.; Jorgensen, R.A.; Allard, R.W. Ribosomal DNA spacer-length polymorphisms in barley: Seeds.

Rosso, M.L.; Shang, C.; Correa, E.; Zhang, B. An Efficient HPLC Approach to Quantify Kunitz Trypsin Inhibitor in Soybean.

Rashed, N.A.; Macdonald, M.H.; Matthews, B.F. Protease Inhibitor Expression in Soybean Roots Exhibiting Susceptible and Resistant Interactions with Soybean Cyst Nematode. J. Nematol. 2008, 40, 138–146.

Riascos, J. A Genomics-Based Search for Novel Soybean Allergens; 2009, doi:10.13140/RG.2.1.3690.0720.

Yuan, J.; Wen, Z.; Gu, C.; Wang, D. Introduction of High Throughput and Cost Effective SNP Genotyping Platforms in Soybean. Plant Genet. Genom. Biotechnol. 2017, 2, 90–94, doi:10.5147/pggb.v2i1.155.

Pati, G.; Chaudhary, J.; Vuong, T.D.; Jenkins, B.; Qiu, D.; Kadam, S.; Shannon, G.J.; Nguyen, H.T. Development of SNP Genotyping Assays for Seed Composition Traits in Soybean. Int. J. Plant Genom. 2017, 2017, 1–12, doi:10.1155/2017/6572969.

Kadam, S.; Vuong, T.D.; Qiu, D.; Meinhardt, C.G.; Song, L.; Deshmukh, R.K.; Patil, G.; Wan, J.; Valliyodan, B.; Scalbo, A.M.; et al. Genomic-assisted phylogenetic analysis and marker development for next generation soybean cyst nematode resistance breeding. Plant Sci. 2016, 242, 342–350, doi:10.1016/j.plantsci.2015.08.015.

Rosso, M.L.; Shang, C.; Correa, E.; Zhang. B. An Efficient HPLC Approach to Quantify Kunitz Trypsin Inhibitor in Soybean Seeds. Crop. Sci. 2018, 58, 1616–1623, doi:10.2135/cropsci2018.01.0061.

Saghai-Maroof, M.A.; Sollman, K.M.; Jorgensen, R.A.; Allard, R.W. Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. Proc. Natl. Acad. Sci. USA 1984, 81, 8014–8018, doi:10.1073/pnas.81.24.8014.

Song, Q.; Hyten, D.L.; Jia, G.; Quigley, C.V.; Fickus, E.W.; Nelson, R.L.; Cregan, P.B. Development and Evaluation of SoySNP50K, a High-Density Genotyping Array for Soybean. PLoS ONE 2013, 8, e54985, doi:10.1371/journal.pone.0054985.

Van Ooijen, J.W. Joinmap 4® Software for the Calculation of Genetic Linkage Maps. In Experimental Populations; Kyazma B; V: Wageningen, The Netherlands, 2006.

Kosambi, D.D. The Estimation of Map Distances from Recombination Values. Annals of Eugenics. Ann. Eugen. 1943, 12, 172–75.

Meng, L.; Li, H.; Zhang, L.; Wang, J. QTL Imapping: Integrated Software for Genetic Linkage Map Construction and Quantitative Trait Locus Mapping in Biparental Populations. Crop J. 2015, 3, 269–283.

Voorrips, R.E. MapChart: Software for the Graphical Presentation of Linkage Maps and QTLs. J. Hered. 2002, 93, 77–78, doi:10.1093/jhered/93.1.77.

Zhu, Q.; Escamilla, D.M.; Wu, X.; Song, Q.; Li, S.; Rosso, M.L.; Lord, N.; Xie, F.; Zhang, B. Identification and validation of major QTLs associated with low seed coat deficiency of natto soybean seeds (Glycine max L.). Theor. Appl. Genet. 2020, 133, 3165–3176, doi:10.1007/s00122-020-03662-5.

Qi, J.; Hyten, D.L.; Jia, G.; Quigley, C.V.; Fickus, E.W.; Nelson, R.L.; Cregan, P.B. Fingerprinting Soybean Germplasm and Its Utility in Genomic Research. G3: Genes Genomes Genet. 2015, 5, 1999.

Nyquist Wyman, E.; Baker, R.J. Estimation of Heritability and Prediction of Selection Response in Plant Populations. Crit. Rev. Plant Sci. 1991, 10, 235–322.