Mutation of GmIPK1 Gene Using CRISPR/Cas9 Reduced Phytic Acid Content in Soybean Seeds

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Abstract: Phytic acid (PA) acts as an antinutrient substance in cereal grains, disturbing the bioavailability of micronutrients, such as iron and zinc, in humans, causing malnutrition. GmIPK1 encodes the inositol 1,3,4,5,6-pentakisphosphate 2-kinase enzyme, which converts myo-inositol-1,3,4,5,6-pentakisphosphate (IP5) to myo-inositol-1,2,3,4,6-hexakisphosphate (IP6) in soybean (Glycine max L.). In this study, for developing soybean with low PA levels, we attempted to edit the GmIPK1 gene using the CRISPR/Cas9 system to introduce mutations into the GmIPK1 gene with guide RNAs in soybean (cv. Kwangankong). The GmIPK1 gene was disrupted using the CRISPR/Cas9 system, with sgRNA-1 and sgRNA-4 targeting the second and third exon, respectively. Several soybean Gnipk1 gene-edited lines were obtained in the T0 generation at editing frequencies of 0.1–84.3%. Sequencing analysis revealed various indel patterns with the deletion of 1–9 nucleotides and insertions of 1 nucleotide in several soybean lines (T0). Finally, we confirmed two sgRNA-4 Gnipk1 gene-edited homozygote soybean T1 plants (line #21-2: 5 bp deletion; line #21-3: 1 bp insertion) by PPT leaf coating assay and PCR analysis. Analysis of soybean Gnipk1 gene-edited lines indicated a reduction in PA content in soybean T2 seeds but did not show any defects in plant growth and seed development.

Keywords: soybean; phytic acid; genome editing; CRISPR/Cas9; sgRNA; GmIPK1

1. Introduction

Soybeans (Glycine max (L.) Merr.) are one of the world’s most important crops, containing oils, proteins, carbohydrates, dietary fibers, vitamins, and minerals. Transgenic soybean, a genetically modified crop, occupies a large portion of genetically modified organism (GMO) fields for its food, nutritional, industrial, and pharmaceutical applications [1–3]. To date, various useful genes with desired traits have been introduced into soybeans using Agrobacterium-mediated transformation, in which transgenic soybean plants have been successfully produced using mature or immature cotyledon explants [4] and improved by incorporating an alternative explant derived from mature soybean seeds [5–9]. Moreover, the addition of various thiol compounds, including dithiothreitol, L-cysteine, and sodium thiosulfate, significantly enhanced transformation efficiency by reducing the oxidative stress that causes tissue browning or cell death in CN explants. The modified thiol compound method also positively affects organogenesis and shoot growth in the shoot pad [6,8,10,11].

With these improved transformation processes, the consequences of foreign gene introduction have resulted in unexpected results due to the disruption of endogenous plant genes or exogenous gene silencing. In particular, RNAi application to the individual gene of interest has silenced entire gene families [12]. Thus, more accurate methods using different strategies, such as genome editing, have been developed. Genome editing rapidly
produced new agronomically desirable traits, such as high oleic acid content, improved plant architecture, and rapid domestication of wild phenotypes with disease resistance and stress tolerance [13–15].

Myo-inositol-1,2,3,4,5,6-hexakisphosphate (IP$_6$), commonly known as phytic acid (PA), is a major phosphorus form in soybean and other plant seeds [16]. In most cereal seeds, PA occurs as phytates in protein bodies, which can strongly chelate cations such as calcium, zinc, magnesium, copper, and iron [17]. Phytase enzymes are a class of phosphatases that catalyze the sequential hydrolysis of PA to less phosphorylated myo-inositol derivatives and inorganic phosphates [18]. During seed germination, endogenous phytase enzymes are activated and hydrolyze PA. This releases bound mineral cations, stored phosphorus (P), and inorganic phosphorus utilized for seedling growth [16]. Monogastric animals cannot utilize PA because of their lack of phytase enzymes. Thus, undigested phytic acid phosphorous (PA-P) in animal waste (manure) is one of the major causes of environmental phosphorus pollution [19,20]. Therefore, there is considerable interest in generating cereal crops with low PA content, such as soybeans, rice, maize, barley, common beans, and wheat.

Most phosphorus (P) in soybean seeds is found in the form of phytate (65–85%), leading to a deficiency in available P in humans consuming soybean seeds [16]. Therefore, soybean meal is usually enhanced with supplemental mineral P or phytase enzymes to increase P availability [21,22]. Twelve genes predicted to encode enzymes in the PA metabolic pathway were identified in cereals. Mutations in these 12 genes through mutagenesis and genetic engineering have been studied as an approach to creating lpa (low phytic acid) mutants in cereals [23,24]. For example, Yuan et al. (2007) previously reported the two soybean lpa mutant lines, Gm-lpa-TW-1 (a 2 bp deletion in the third exon of GmMIPS1) and Gm-lpa-ZC-2 (a G-A point mutation of Gmipk1) generated through gamma irradiation and EMS treatment, exhibited PA reductions of 66.6 and 46.3%, respectively [25]. Additionally, the Gm-lpa-TW-1 mutant showed a significantly reduced field emergence rate, whereas the Gm-lpa-ZC-2 mutation did not negatively affect seed viability and yield traits.

Inositol-pentakisphosphate 1-kinase (IPK1) converts 1,3,4,5,6-pentakisphosphate (IP$_5$) to PA and catalyzes the last step of PA biosynthesis. The soybean genome contains three IPK1 homologs on chromosomes 14, 6, and 4 (Glyma14g07880, Glyma06g03310, and Glyma04g03240 in Glycine max v1.1 [3]. GmIPK1 (Glyma14g07880) was highly expressed in immature soybean seeds compared with the other two genes [26]. The Gm-lpa-ZC-2 mutant (Gmipk1) dramatically decreased the PA content by up to approximately 50% compared with wild-type seeds. Therefore, we attempted to disrupt GmIPK1 via genome editing of the CRISPR/Cas9 system to reduce PA content in soybean. The creation of targeted mutation by genome editing is a more applicable and accurate way than mutagen treatment. In this study, we generated transgenic soybean plants with genome-edited GmIPK1 sgRNA-1 and sgRNA-4, which target different sites in the soybean GmIPK1 gene. CRISPR/Cas9-mediated genome editing technology with two guide RNAs decreased the levels of PA in T$_2$ genetically engineered plant seeds. Our results suggest that genome editing can be a precise tool to produce new crops by decreasing targeted metabolites in plants.

2. Results and Discussion

2.1. Generation of Gmipk1 Gene-Edited Soybean Plants by CRISPR/Cas9 System

A previous study reported that GmIPK1 (Glyma14g07880) was highly expressed in seeds compared with other homologous genes Glyma06g03310 and Glyma04g03240 in Glycine max L. var. Pusa 9712 [26]. Thus, we first examined their expression levels by qRT-PCR in the following organs: leaves, stems, roots, flowers, seed pods, and seeds at two vegetative stages (VC and V2) and three reproductive stages (R2, R6, and R7) of the Korean soybean cultivar Kwangankong. High-level expression of GmIPK1 and its two homologs was detected in the stems, roots, and seed pots of the R6 (full seed) stage and the seeds of the R7 (beginning maturity) stage. The highest expression levels were observed in the stems of the R6 stage and the seeds of the R7 stage. In particular,
**GmIPK1** exhibited a significantly higher expression level relative to the two homologs (Figure 1). This suggests that GmIPK1 may play an important role in seed development and maturation. Previous studies also reported that IPK genes from rice and wheat had high expression levels at the seed development and maturation stages [27,28]. As the expression level of GmIPK1 (Glyma14g07880) was significantly higher in the seed development and maturation compared with Glyma06g03310 and Glyma04g03240, GmIPK1 was selected as a target gene for gene editing to develop low phytic acid soybean plants.

To isolate GmIPK1 homologs in Kwangankong, PCR was performed with gene-specific primers for GmIPK1 (Glyma14g07880) using cDNA and genomic DNA as templates. The amplified GmIPK1 cDNA sequence contained an open reading frame of 1371 bp, encoding a protein of 456 amino acid residues. Nucleotide sequence analysis showed that the amplified GmIPK1 cDNA and genomic sequences in Kwangankong had 100% similarity to the Glyma04g03240 and Glyma06g03310 sequences from the soybean genome [29] (data not shown).

![Expression analysis of GmIPK1 (Glyma14g07880) (white bar) and its homologous genes Glyma06g03310 (gray bar) and Glyma04g03240 (black bar) in different tissues and organs of soybean (cv. Kwangankong).](image)

**Figure 1.** Expression analysis of GmIPK1 (Glyma14g07880) (white bar) and its homologous genes Glyma06g03310 (gray bar) and Glyma04g03240 (black bar) in different tissues and organs of soybean (cv. Kwangankong). Relative expression levels were measured by qRT-PCR analysis following normalization with GmPEPCo gene control. The data are the mean of three technical replicates (SD). For qRT-PCR analysis, each sample was harvested at two vegetative stages (VC and V2) and three reproductive stages (R2, R6, and R7) of soybean (cv. Kwangankong) plants.

To obtain soybean genome-edited plants with a low concentration of PA, four target sites (referred to as target 1, target 2, target 3, and target 4) were selected with PAM sequence 5'-NGG-3' at the 5' or 3' ends of the second, third, and fifth exons of GmIPK1 (Figure 2A). The mutagenesis of soybean protoplasts was performed using 40% PEG-mediated transformation with pJY_GmU6-10_SpCas9_PPT<sup>R</sup>-sgRNAs (sgRNA-1, sgRNA-2, sgRNA-3, and sgRNA-4). Since the GmlU6-10 promoter has high transcriptional activity and mutation efficiency in soybean hairy roots and *Arabidopsis thaliana* [30], we used the GmlU6-10 promoter instead of the AtU6 promoter to control GmIPK1 sgRNA expression.
To obtain soybean genome-edited plants with a low mutation rate, we used the CRISPR/Cas9 system (Figure 2B). In soybean protoplasts, mutation efficiency was calculated as the percentage of indels detected at the Cas9 cleavage site based on targeted deep sequencing. Because sgRNA-1 and sgRNA-4 induced mutations (0.8% and 0.5% indel frequency, respectively), we selected these two sgRNAs for the subsequent soybean transformation procedure (Figure 3C).

Two CRISPR/Cas9 vector constructs, pJY_GmU6-10_SpCas9_Bar:GmIPK1 sgRNA-1 (referred to as GmIPK1 sgRNA1) and pJY_GmU6-10_SpCas9_Bar:GmIPK1 sgRNA-4 (referred to as GmIPK1 sgRNA4), were used for Agrobacterium-mediated transformation in the Korean soybean cultivar Kwangankong based on the half-seed method with minor modifications (Figure 2B). Agrobacterium-mediated soybean transformation was established in our laboratory by combining the cotyledonary-node (CN) method [4] with alternative mature soybean seeds. This protocol has enabled the production of stable transgenic soybean plants. Moreover, a few modifications, such as the addition of thiol compounds (a mixture of L-cysteine, sodium thiosulfate, and dithiothreitol) to the cocultivation medium, had a positive effect on soybean transformation efficiency [5,6,8,9]. The soybean seeds were inoculated by applying a high concentration of binary vector-containing Agrobacterium solution to a wounded target area (Figure 3A, lane a). Because of the 10 mg L\(^{-1}\) PPT reaction in the shoot induction stage, most primary shoots turned yellow and became necrotic. Elongated shoots with relatively large leaves affected healthy root formation, and the plants were well acclimatized in small pots and grown in the greenhouse (Figure 3A, lanes b–g). PPT leaf coating was performed to confirm herbicide resistance in leaves from nontransgenic (NT) and transgenic plants. Five days after the treatment, the leaves of

**Figure 2.** CRISPR/Cas9-targeted mutagenesis of GmIPK1 in soybean protoplasts. (A) Schematic diagram of GmIPK1 gene structure and four guide RNA target sites used in this study (red arrows). Based on a comparison with GmIPK1 cDNA, the coding regions (4158 bp) of the GmIPK1 gene are divided into seven exons interrupted by six introns. (B) The vector pJY_GmU6-10_SpCas9_Bar was used for CRISPR/Cas9-mediated soybean gene editing with guide RNAs: AtRPS5A\(^{\Delta}\), Arabidopsis AtRPS5A promoter; GmU6-10\(^{\Delta}\), soybean U6-10 promoter; sgRNA, single guide RNA; Nos\(^{\Delta}\), nopaline synthase terminator; SpCas9, human codon-optimized S. pyogenes Cas9; NLS, nuclear location signal; Bar, selective marker gene. (C) Four sgRNA sequences of GmIPK1 are shown. Mutation rates tested in soybean protoplasts were determined by indel frequencies (%) through deep sequencing at target regions of the GmIPK1 gene.
the NT plants turned yellow and reacted to PPT, whereas the transgenic leaves showed resistance to herbicides (Figure 3A lane h). Based on the PPT leaf coating assay results, the transformation efficiencies were approximately 2.9% and 6.1% with GmIPK1 sgRNA-1 and GmIPK1 sgRNA-4, respectively, and, in total, 15 and 25 transgenic plants were produced, respectively (Figures 3B and 2C).

**Figure 3.** Production of Gmipk1 gene-edited soybean transgenic plants via Agrobacterium-mediated transformation. (A) Generation of CRISPR/Cas9-mediated Gmipk1 gene-edited transgenic soybean plants. (a) Cocultivation of half-seeds after inoculation stage (left) and at 5 days after inoculation (right). (b) Shoot induction on shoot induction medium (SIM) without PPT selection for 14 days. (c) Shoot induction on SIM with 10 mg L\(^{-1}\) PPT for another 14 days. (d) Shoot elongation on shoot elongation medium (SEM) with 5 mg L\(^{-1}\) PPT selection. (e) Root formation on rooting medium (RM). (f) Acclimation of a putative transgenic plant in a small pot. (g) Transgenic plant (T\(_0\)) grown in a greenhouse. (h) Leaf coating with 100 mg L\(^{-1}\) PPT showing sensitivity in the leaf of the NT plant (left) and resistance in the leaf of the transgenic plant (right). (B) GmIPK1 sgRNA-1 transgenic soybean plants (T\(_0\)) grown in the greenhouse. (C) GmIPK1 sgRNA-4 transgenic soybean plants (T\(_0\)) grown in the greenhouse.

2.2. Integration and Expression of Transgenes in Transgenic Soybean Plants

To confirm the integration of transgenes in transgenic soybean plants, genomic DNA was isolated from 12 healthy and well-grown GmIPK1 sgRNA-1 T\(_0\) plants and 16 GmIPK1 sgRNA-4 T\(_0\) plants and analyzed via PCR (Figure 4). PCR analysis of GmIPK1 sgRNA-1 transgenic soybean plants showed that the GmIPK1 region, the selectable marker (Bar gene), and the SpCas9 gene were amplified in all 12 transgenic lines as 718 bp, 548 bp, and 4170 bp fragments, respectively (Figure 4A). The GmIPK1 region, Bar gene, and SpCas9 gene were also verified in all 16 transgenic lines from GmIPK1 sgRNA-4 (Figure 4B). Successful amplification of the GmIPK1 region (718 bp) must be from the endogenous GmIPK1 gene.
The copy number of transgene insertions in transgenic soybean plants (T₀) was determined by Southern blot analysis of transgenic lines with sufficient leaf samples using the Bar probe. Four transgenic lines (#6, #10, #12, and #13) seem to have a single insertion, and five transgenic lines (#2, #3, #4, #5, and #7) have multiple copies of the transgene from the result of GmIPK1 sgRNA-1. Among GmIPK1 sgRNA-4 transformants, seven transgenic lines (#1, #2, #5, #13, #20, #21, and #22) seem to have low copy number of the transgene (data not shown).

The expression levels of the transgenes were analyzed in 12 GmIPK1 sgRNA-1 and 13 GmIPK1 sgRNA-4 transgenic soybean plants (T₀) using RT-PCR because of the availability of tissue samples (Figure 5). The Bar gene was expressed in all 12 GmIPK1 sgRNA-1 transgenic lines (Figure 5A). As expected, the expression of GmIPK1 was detectable in all plants, including the nontransformed plants. Expression of the GmIPK1 and Bar genes was also detected in all 13 GmIPK1 sgRNA-4 transgenic lines (Figure 5B). Because of plant culture conditions, we obtained leaf samples from nine mutant lines (T₀) induced by sgRNA-1 and six mutant lines induced by sgRNA-4. Next-generation sequencing (NGS) was conducted with T₀ genomic DNA (sgRNA-1 #2, #3, #4, #5, #6, #7, #10, #12, and #13; sgRNA-4 #1, #2, #3, #5, #20, and #21). They all exhibited mutations with various insertion/deletion (indel) patterns at the GmIPK1 gene target site (Tables S1 and S2; Figures S1 and S2). The sgRNA-1 and sgRNA-4 induced indel mutations in GmIPK1 at efficiencies ranging from 0.1 to 84.3% and from 0.3 to 82.5%, respectively. However, the sgRNA-4 Gmipk1 #21 line had the highest indel frequency (82.5%) and two major indel patterns: a five base deletion and a single base (A) insertion. Thus, we could expect to easily obtain homozygote lines in the next generation (T₁).
Figure 5. Analysis of transcript levels of introduced genes in Gmipk1 gene-edited soybean plants (T₀) using RT-PCR. Total RNA was extracted from GmIPK1 sgRNA-1 and GmIPK1 sgRNA-4 transgenic soybean plants (T₀), and RT-PCR was used to confirm the transgene expression. GmTUB gene was used as a quantitative control: NT, nontransgenic plant; (A) GmIPK1 sgRNA-1 transgenic lines (#1–#10, #12, #13); (B) GmIPK1 sgRNA-4 transgenic lines (#1–#5, #7, #12, #15–#16, #18, #20–#22).

2.3. Selection of CRISPR/Cas9-Induced sgRNA-4 Gmipk1 Gene-Edited Line and Measurement of PA Content

To check the heritability of Gmipk1 mutations, T₁ seeds were collected from all sgRNA-1 or sgRNA-4 Gmipk1 mutants. Four T₀ plants (lines #4 and #12 for sgRNA-1 and lines #20 and #21 for sgRNA-4) with high indel frequencies were selected to obtain T₁ seeds and analyze gene editing patterns in the T₁ generation. Ten seeds from each self-pollinated T₀ plant were grown in a growth chamber, and a total of 24 T₁ plants were obtained because of successful germination (sgRNA-1 #4 (5 lines), #12 (3 lines); sgRNA-4 #20 (5 lines), #21 (11 lines)). Indel frequencies for sgRNA-1 Gmipk1 and sgRNA-4 Gmipk1 gene-edited plants (T₁) displayed various patterns, with different percentages of total mutation reads. Consistent with the deep sequencing results of T₀ plants, the progenies of T₁ plants showed five base deletions in Gmipk1 #21-2 line and a single base insertion (A) in Gmipk1 #21-3 with 100% indel frequency (Figure 6A). Other GmIPK1 #21 T₁ lines (#21-1, #21-4, #21-5, #21-6, #21-11) showed two types of mutations, half and half percentages, similar to the GmIPK1 #21 T₀ lines. Although T₁ lines derived from Gmipk1 #4, #13 (sgRNA-1), and #20 (sgRNA-4) had a high percentage of indel frequency, it was difficult to obtain homozygous mutant lines because the patterns varied (data not shown). Thus, we used Gmipk1 #21-2 and #21-3 mutant lines for further experiments in this study. Both Gmipk1 gene-edited lines had a premature stop codon almost immediately after the mutation sites. However, the T₁ plant growth of Gmipk1 #21-2 and #21-3 was comparable to that of the NT controls (data not shown). Significant differences in plant height and morphological phenotype were not observed between Gmipk1 #21-2 and #21-3 mutants compared with NT controls. Similarly, Yuan et al. (2012) reported that the Gmipk1 mutant (Gm-lpa-ZC-2) displayed low PA content in seeds and had no adverse effects on seed viability and agronomic traits in soybean. Therefore, we also measured total and free phosphorus (Pi) levels in the Gmipk1 #21-2 and #21-3 mutants to determine whether they exhibited a reduction in PA content. Significant decreases of 20.7% and 25.7% in PA content were observed in T₂ seeds obtained from Gmipk1 #21-2 and #21-3 mutant lines, respectively (Figure 6B). To check potential off-target events by sgRNA-4 in homologous genes Glyma06g03310 and Glyma04g03240,
possible sgRNA-4 target sites were analyzed in the two homologous genes. Five nucleotide sequences were different from those of the sgRNA-4 target site in Glyma14g07880 (Table S3). Genomic DNA was extracted from the leaves of Gmipk1 #21-2 and #21-3’s T2 generation (four plants generated by #21-2 and two plants generated by #21-3), and then the potential off-target sites were amplified by PCR with gene-specific primers for sequence analysis. No mutations were found in the two homologous genes of six Gmipk1 gene-edited plants, thus indicating that sgRNA-4 targeted only Glyma14g07880 but not in its homologs and induced mutagenesis of GmIPK1 through the CRISPR/Cas9 system (Table S3).

![Figure 6](image-url)

**Figure 6.** Inheritability of CRISPR/Cas9-induced mutations in Gmipk1 gene-edited transgenic soybean plants. (A) Indel mutation patterns and ratios in T1 progenies of T0 plant. The mutation ratio (Indel %) was calculated by dividing the number of reads containing indels at the target site (Reads #) by the number of total sequencing reads. The protospacer adjacent motif (PAM) sequences (NGG) are bold and underlined. Insertions and deletions are represented by red font and green hyphens, respectively. (B) Total phytic acid contents in T2 seeds of soybean Gmipk1 gene-edited lines #21-2 and #21-3. Phytic acid was measured in the mature T2 seeds from each GE line #21-2 and #21-3. The symbols ** and *** indicate significant differences at *p* = 0.001 and 0.0001, respectively (*n* = 3).

In this study, we demonstrated the efficient reduction in PA content in soybean seeds through CRISPR/Cas9 gene editing of GmIPK1. Recently, efforts have been made to develop low PA plants in soybean by silencing the regulatory genes involved in the biosynthesis of PA, such as GmMIPS1 and GmIPK2 [31,32]. GmMIPS1 and GmIPK2 genes encode DG-myoinositol-3-phosphate synthase 1 and myo-inositol polyphosphate 6-/3-/5-kinase 2, respectively. Kumar et al. (2019) reported that RNAi triggered seed-specific silencing of GmMIPS1 as the target gene exhibited a 41% reduction in phytate content without damaging growth and seed development. Similarly, Punjabi et al. (2018) reported that the disruption of GmIPK2, the upstream gene of GmIPK1 in the PA biosynthetic pathway, as the target gene by RNAi in the seeds showed low PA levels, moderate accumulation of inorganic phosphate, and elevated mineral content in some transgenic lines. These results suggest that PA reduction in RNAi transgenic soybean seeds did not show any significant abnormal agronomic traits, including seed germination and development. In the present study, CRISPR/Cas9-
mediated gene-edited soybean plants with the GmIPK1 gene resulted in an approximately 25% reduction in PA content without affecting seed development and plant growth. Based on previous reports and our results, we speculate that mutations in two or three genes such as GmMIPS1, GmIPK2, and GmIPK1 could reduce PA levels by around 40–50% in seeds without any growth and development defects. To further investigate the agronomical traits of Gmipk1 gene-edited lines, we are currently growing them in the GMO field. After looking into yield components and germination rates of Gmipk1 gene-edited plants, the effect of reduced PA will be clarified.

In this study, GmIPK1 (Glyma14g07880) was highly expressed in the seed developmental stage compared with the other homologs, Glyma06g03310 and Glyma04g03240, in cv. Kwangankong (Figure 1). Therefore, we first focused on GmIPK1 (Glyma14g07880) to generate soybean plants with low PA levels. Because Glyma06g03310 and Glyma04g03240 had extremely high sequence identity (96.65%) at the nucleotide sequence level, and their sequence similarity with GmIPK1 was 80.90% and 80.75%, respectively, it was difficult to design one specific sgRNA that targeted all the GmIPK1 homologs. Therefore, as the next step to obtain gene-edited soybean plants with a much lower PA content, we are currently generating CRISPR/Cas9-mediated soybean gene-edited plants using one specific sgRNA that simultaneously targets the two GmIPK1 homologs (Glyma06g03310 and Glyma04g03240). Subsequently, it will be crossed with the Gmipk1 #21-2 and #21-3 lines.

3. Materials and Methods

3.1. RNA Isolation and Quantitative Real-Time Reverse Transcription PCR (qRT-PCR)

Expression patterns of Glyma14g07880, Glyma06g03310, and Glyma04g03240 were analyzed in soybean various tissues and organs at different development stages by qRT-PCR. Soybean samples were kindly provided by Dr. Man-Soo Choi (National Institute of Crop Science, Rural Development Administration, Wanju, Korea). Total RNAs were isolated from the tissues and organs of different growth stages of the Korean soybean cultivar Kwangankong using the Ribospin Plant (GeneAll, Seoul, Korea), and cDNAs were synthesized using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer’s protocols. Quantitative PCR was performed using BioFACT 2X Real-Time PCR Series (with SFCgreen I) (BioFACT, Daejeon, Korea) according to instructions for the CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA, USA). The relative expression levels were calculated using the comparative threshold method. The soybean PEPC carboxylase gene (GmPEPCo) was used as the reference gene to normalize all data (qGmPEPCo forward primer 5′-CATGCACCAAAGGGTGTGTTGGGTTT-3′ and reverse primer 5′-TTTTGCGGCAGCTATCTCTC-3′). The primers of Glyma14g07880, Glyma06g03310, and Glyma04g03240 were utilized to amplify the 200 bp fragment (qGlyma14g07880 forward primer 5′-GACGCAGCTGACTGGGTTTA-3′ and reverse primer 5′-GTCATCTGTAAGAGCCTGCTAAATCTC-3′) [26,33].

3.2. Construction of Two Gmipk1 Genome Editing Vectors for Soybean Transformation

The GmIPK1 (Glyma14g07880) cDNA was amplified using the first-strand cDNA of total RNA from the developing seeds of Kwangankong as a template for polymerase chain reaction (PCR) with GmIPK1 primers (forward primer 5′-ATGGCATTGACTTTGAAAGG-3′ and reverse primer 5′-CATGACCAAAGGGTGTGTTGG-3′). The PCR products were purified by gel purification using the Expin Gel SV kit (GeneAll, Seoul, Korea) and cloned into the All One PCR cloning vector (BioFACT, Daejeon, Korea). Finally, GmIPK1 cDNA was confirmed by DNA sequencing and comparison with the GmIPK1 sequences from the soybean Wm82.a22.v1 genome on JGI Phytozome 13 (http://phytozome-next.jgi.doe.gov/, accessed on 25 June 2020) [29].
Target sequences were identified in the coding region of the GmIPK1 gene to design unique sgRNAs. Four guide RNA sequences (sgRNA-1, sgRNA-2, sgRNA-3, and sgRNA-4) containing a protospacer adjacent motif (PAM) sequence were selected using the Cas-Designer site (http://www.rgenome.net/cas-designer/, accessed on 27 April 2021). To verify the efficiency of GmIPK1 sgRNAs, we constructed a pJY_GmU6-10_SpCas9_Bar plasmid vector expressing Streptococcus pyogenes Cas9 (SpCas9) and GmIPK1 sgRNAs in a CRISPR/Cas9 system [34]. The SpCas9 gene was expressed under the control of the AtRPS5A promoter in the pJY_GmU6-10_SpCas9_Bar plasmid, and the soybean U6-10 promoter (GmU6-10) was used to drive GmIPK1 sgRNA expression in soybean plants [30]. Additionally, the Bar gene was used as a selectable marker gene for modified phosphinothricin (PPT).

Protoplasts were extracted from the unifoliate leaves of soybean plants by incubation with 3x valosin-containing protein (VCP) enzymes for 12 h at room temperature. PEG-mediated RNP delivery was performed as previously described [35] with minor modifications. Briefly, 2 × 10^5 protoplasts were mixed with preassembled Cas9/gRNA (1:5 molar ratio) in 150 µL of MMg (4 mM MES, 0.4 M mannitol, and 15 mM MgCl2) via an equal volume of freshly prepared PEG solution (40% (w/v) PEG 4000, 0.2 M mannitol and 0.1 M CaCl2). Transfected protoplasts were kept at 22 °C for 2 days and collected for indel frequency analysis. The two desired plasmids, pJY_GmU6-10_SpCas9_PPT\textsuperscript{R}:GmIPK1-1 and pJY_GmU6-10_SpCas9_PPT\textsuperscript{R}:GmIPK1-4, were transformed into Agrobacterium tumefaciens EHA105 [36] and used for soybean transformation.

3.3. Next-Generation Sequencing (NGS) Analysis

Genomic DNA was isolated from the leaves of gene-edited T\textsubscript{0} and T\textsubscript{1} plants using the cetyltrimethylammonium bromide (CTAB) procedure. Targeted regions of SpCas9 and sgRNA complexes were amplified by three rounds of PCR. First, the genomic region containing the guide RNA-binding sites was amplified to approximately 500 bp. First-time PCR products were diluted 1:10 and used as templates for the second PCR. Illumina adaptors and barcode sequences were added to the second and third PCR products. The final PCR products were quantified using a NanoDrop (Thermo Scientific, Waltham, MA, USA), and targeted deep sequencing was supported by Bio Core facilities in KAIST (http://biocore.kaist.ac.kr, accessed on 15 July 2021). Indel frequencies and mutation patterns were analyzed using a CAS-Analyzer in RGEN tools [37].

3.4. Agrobacterium-Mediated Soybean Transformation

Stable transgenic soybean plants were generated using mature soybean seeds of the Korean cultivar Kwangankong, following the half-seed method with modifications described previously [6,7]. Three batches of transformation experiments were carried out with 130–150 soybean seeds per batch for soybean transformation. The BASTA herbicide was used to coat the upper surface of two trifoliate leaves of transgenic (T\textsubscript{0}) plants with a mixture of 100 mg L\textsuperscript{-1} DL-phosphinothricin (PPT) and Tween-20 to screen putative transformants expressing the Bar gene. The response to the herbicide was observed 3–5 days after PPT application. Plants with undamaged and herbicide resistance were selected as putative transformants, continuously grown in a greenhouse, and the seeds were harvested.

3.5. Confirmation of Transgenes in Transgenic Soybean Plants (T\textsubscript{0})

Genomic DNA was extracted from leaf tissues of nontransgenic (NT) and transgenic (T\textsubscript{0}) plants using CTAB. To detect transgenes in soybean plants, PCR was conducted using Prime Taq Premix (2X) (GeNet Bio, Daejeon, Korea) according to the manufacturer’s instructions. The primer sets were designed to amplify specific regions of GmIPK1 (forward primer 5\textsuperscript{′}-TCCGTGCGCTGTGCTGCGCTG-3\textsuperscript{′} and reverse primer 5\textsuperscript{′}-GAATGATCTGACATGAGAAG-3\textsuperscript{′}) and Bar (forward primer 5\textsuperscript{′}-ATGAGCCCCAACAGCGCCCGGGCC-3\textsuperscript{′} and reverse primer 5\textsuperscript{′}-GGGTCATCAGATTTCCGTGACGGG-3\textsuperscript{′}). Amplification products of 718 bp and 548 bp,
respectively, were expected. The inserted SpCas9 gene was also amplified by dividing it into three parts designated as SpCas9-a (forward primer 5′-ATGGACAAGAAGTACAGCATCGGC-3′ and reverse primer 5′-AATCTGTGAACCTCCTGGCTG-3′), SpCas9-b (forward primer 5′-CAGATCGGCAGACAGTACGC-3′ and reverse primer 5′-AGAAGTGAGCTCTGGGGAAGT-3′), and SpCas9-c (forward primer 5′-CTGACGGAGCTGACAAAAGGGCGG-3′ and reverse primer 5′-TTAGGCGTAGTCGGCCACGTTCGTA-3′). Amplification products of 1124 bp, 2077 bp, and 1449 bp, respectively, were expected.

3.6. RNA Analysis of Transgenic Plants (T₀)

Total RNA was isolated from NT and T₀ plants using Plant RNA Purification Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. To confirm gene expression in soybean plants, reverse transcriptase PCR (RT-PCR) was conducted using the SuPrimeScript RT-PCR Premix (2X) (Genet Bio, Daejeon, Korea) according to the manufacturer’s instructions. The primer sets for GmIPK1 (forward primer 5′-TCCGTTGCTTGTTGTAGCTG-3′ and reverse primer 5′-GAATGATCTGACATGAGAAG-3′) and Bar (forward primer 5′-ATGAGCCCAGAACGACGCCCGGCC-3′ and reverse primer 5′-GGTACATCAGATTTCCGTGACGGG-3′) were used. The constitutive TUB gene (forward primer 5′-TGACGACTTCGACCCGACGGG-3′ and reverse primer 5′-CTCCGGACAGTGCACTCCTG-3′) was used as the internal control to normalize the amount of RNA [38].

3.7. Selection of Gmipk1 Gene-Edited Soybean Plants and Generation Advance

An herbicide assay was performed to determine whether the selectable marker Bar gene was knocked out in Gmipk1 gene-edited soybean plants. NT and T₁ seeds were planted in a seedling tray, and the upper surface of two trifoliate leaves was coated across the midrib with 100 mg L⁻¹ DL-phosphinothricin (PPT) mixed with Tween-20 using a brush. The response to the herbicide was observed 3–5 days after PPT application. Herbicide-sensitive and herbicide-resistant plants were separately grown in a greenhouse to obtain T₂ seeds.

3.8. Determination of PA Content

Measurement of total PA in mature seeds of the T₂ and nontransgenic lines was performed using the Megazyme PA/Total phosphorus kit (Megazyme Inc., Bray, Ireland) according to the manufacturer’s protocol. Briefly, the mature seeds were ground to a fine powder, and extraction was performed using 0.66 N hydrochloric acid with vigorous stirring for 10–12 h at ambient temperature. This method includes acid extraction of inositol phosphates and further treatment with phytase and alkaline phosphates to free phosphates. The supernatant was used for the colorimetric assay according to the manufacturer’s instructions.

4. Conclusions

We developed soybean gene-edited plants with a low PA content using the CRISPR/Cas9 system to mutate the GmIPK1 gene. The mutation of GmIPK1 led to a reduction in PA content in soybean seeds but did not show any growth defects or seed viability in soybean plants. Furthermore, identifying the superior alleles of GmIPK1 would represent valuable resources for the genetic improvement of soybean plants with low PA levels.

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