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Research Article

Efficient Designing, Validation, and Transformation of *GmIPK2* Specific CRISPR/Cas9 Construct for Low-Phytate Soybean

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Abstract

Background: The nutritive potential of soybean is limited by the presence of an absorption inhibitor – phytic acid. Development of low-phytate soybean has been envisioned; but, limited variations in the gene pool and the regulatory hurdles have handicapped this process. The present study uses type II CRISPR/Cas9 system for precise editing of *GmIPK2* (inositol polyphosphate 6-/3-/5-kinase).

Methodology: Single guide RNA (sgRNA) sequences were designed and validated for their efficiency by using various webtools (CRISPR-scan, RNA fold server, and Cas-OFFinder). A single binary vector carrying the guide RNA and Cas9 cassette was designed and expressed transiently in soybean leaf discs by using AGRODATE (*Agrobacterium*-mediated Disc Assay for Transient Expression) method to edit the *GmIPK2* gene. We observed deletions of 2 to 5 nucleotides in the target region of the analyzed leaf discs; thus, validating efficacy of the construct *in vivo*. Stable transformation of soybean with Cas9/gRNA-*GmIPK2* construct was also carried out.

Conclusion: The experimental sensitivity resulting from sgRNA efficiency is a major hurdle in successful CRISPR/Cas9-based genome editing. Employing multiple webtools and use of transient expression assays as depicted in this study can speed-up the CRISPR/Cas9 based editing in recalcitrant crops like soybean.

Keywords: CRISPR/Cas9; Genome Editing; Soybean; Phytic Acid; Crop Improvement

Introduction

Soybean is the fourth most highly cultivated crop in the world [1]. It has excellent fatty acid content, unmatched protein quality, abundance of vitamins, minerals, dietary fibre, and nutraceuticals [2]. The higher cultivation and production of this crop, together with the exceptional nutrient profile, make it an ideal candidate for ensuring both food and nutritional security. However, high content (4 - 5%) of phytic acid hampers the uptake of minerals and proteins from soybean in organisms lacking phytase enzyme [3]. Raw soybean contains 15.7 mg Iron, 4.89 mg Zinc, and 277 mg Calcium per 100g seeds, of which only 8.03-9.1% Iron, 7.32-8.9% Zinc, and 8.1-9.13% Calcium is bioavailable [4]. Phytic acid also interacts with basic amino acids, seed proteins, and enzymes in the digestive tract reducing amino acid availability [5], protein digestibility and activity of digestive enzymes [6,7]. Unutilized phytic acid is excreted in manure causing phosphorus runoff leading to eutrophication [8].

Low-phytate crops can solve this problem at the root, by improving phosphorous availability and utilisation while reducing phosphorous excretion and its consequences [9]. Moreover, low-phytate food can cause 30-50% increase in bioavailability of minerals [10].

Phytic acid production in soybean is a multi-step process (Figure 1) that converts glucose-6-phosphate into *myo*-inositol hexa*kis*phophate i.e., phytate [11]. Targeting the early pathway enzymes often resulted in the plant having poor agronomic characteristics due to the numerous roles played by inositol phosphates [12]. Hence, manipulating the late pathway enzymes to reduce phytate, while allowing synthesis of lower inositol phosphates is a propitious solution. The gene targeted for manipulation in this study is *GmIPK2* (Inositol polyphosphate 6-/3-/5-kinase), which catalyses reactions leading to synthesis inositol pentaphosphate, the immediate precursor of phytic acid. Seed-specific silencing of *GmIPK2* using RNAi caused 45% reduction in phytic acid levels and 39% increase in inorganic phosphate content, without altering the mean total phosphorous content [13]. Hence, targeting this gene would only affect phosphorous partitioning and allow the plant to strive by balancing the P-related mechanisms in the seed.

Figure 1: Pathway depicting the critical enzymes involved in both lipid -independent and lipid-dependent routes of phytic acid biosynthesis in soybean. IPK2 (inositol polyphosphate 6-/3-/5-kinase) catalyzing steps are highlighted.

Bypassing the impediments of classical breeding techniques and the regulatory issues of RNAi-based transgenics, the present study was carried out using the genome-editing tool CRISPR/Cas9 which has higher social acceptance owing to the fact that transgenic Cas9 gene and the antibiotic-resistant marker genes can easily be removed by segregation in the progenies, resulting in transgene-free plants (null segregants) [14]. Cas9-mediated genome editing requires: a protospacer-adjacent motif (PAM) [15] in the genomic sequence, immediately downstream of the targeted DNA; a single guide RNA (sgRNA) consisting of the target sequence (20 nt) at the 5′-end; a conserved 3′-end scaffold with a special stem-loop structure that binds Cas9 and; a Cas9 protein with a nuclear localization signal [16]. Complex formation between sgRNA and Cas9 brings about conformational changes that activates Cas9 for binding onto dsDNA [17] and for searching PAM sequences in the genomic DNA. Once PAM is detected, Cas9 separates the DNA duplex and checks for complementarity to the guide sequence. Upon successful match, Cas9 generates a blunt ended double-stranded break (DSB) [18]. Cells commonly repair DSBs by the error-prone NHEJ pathway resulting in insertions/deletions having mutagenic potential [19]. Cas9/sgRNA nuclease complex can be easily programmed to target sites by introducing the target sequences into the sgRNA [20]. This feature makes CRISPR/Cas9 system facile, rapid, and cost-effective.

Since specificity and efficiency are crucial in CRISR/Cas9-based editing, we used webtools to design a CRISPR/Cas9 construct containing sgRNA sequence with a higher probability of causing gene disruption. Additionally, we conducted transient expression assay by AGRODATE method [21] for a quick double-check of the sgRNA efficacy before proceeding to the tedious process of stable transformation. After establishing the efficacy of the construct, stable *Agrobacterium*-mediated transformation of soybean was done by cotyledonary node method [22]. The steps involved in developing a CRISPR-edited transgene-free plant is illustrated (Figure 2).

Materials and Methods

Plant material

Soybean seeds (*Glycine max* L. Merr. cv DS-9712), procured from the Division of Genetics, ICAR-IARI, New Delhi, India was sown in pots and maintained in the National Phytotron Facility (NPF) at 28°C, 10:14 h photoperiod condition for a duration of 7 weeks for transient analysis. Seeds of the same genotype were also used for genetic transformation.

Selection of target-specific sgRNA sequences

The *GmIPK2* gene sequence (Accession no. KF297702) was retrieved from NCBI and used as input sequence for Stupar's CRISPR design webtool (http://stuparcrispr.cfans.umn.edu/CRISPR/), yielding seven potential sgRNA spacer sequences with 5' NGG 3' PAM (Protospacer Adjacent Motif) at the 3'end. These sgRNAs were

analysed for off-target activity by using Cas-OFFinder tool (http://www.rgenome.net/cas-offinder/) and NCBI-BLAST to confirm the uniqueness of target sequences in the soybean genome (www.phytozome.org). Based on the analysis, three of the seven sgRNA spacer sequences were selected and examined for cleavage efficiency by analysing the GC content and Minimum Free Energy (MFE) by using OligoAnalyzer (https://www.idtdna.com/pages/tools/oligoanalyzer) and RNAfold Server (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi) respectively. Stability and activity of sgRNAs was also studied by using the online tool CRISPRscan (https://www.crisprscan.org/). Finally, the best sgRNA spacer sequence was selected for construction of Cas9/gRNA-*GmIPK2* vector.

Figure 2: Flowchart depicting steps for generating CRISPR-edited transgene-free plants. (a) Target selection for sgRNA, (b) Generation of CRISPR/Cas9 construct, (c) Transient expression analysis of the construct, (d) Generation of stable transformants, (e) Confirmation of mutagenesis in the transformed plants by using restriction enzyme site-loss assay or sequencing methods, (f) Biochemical and phenotypical evaluation of the CRISPR-edited plants, (g) Use of breeding techniques to identify null-segregants, and (h) Select for CRISPR-edited transgene-free plants.

Construction of the Cas9/gRNA-*GmIPK2* vector

The vector construction was done based on protocol developed by Stupar's lab. Forward and reverse primers (Table 1) specific to target site were designed with *BbsI* overhangs at the 5'end and annealed at 50°C for 6 h followed by cloning into *BbsI* sites of pBlu/gRNA vector (Addgene plasmid ID 59188). The cloning was confirmed by restriction analysis with *StyI* and BLAST analysis of the vector sequences. The recombinant vector (pBlu/gRNA-*GmIPK2*) containing U6 promoter upstream and scaffold RNA downstream of the target oligos was then subjected to restriction analysis by using *EcoRI* to isolate the AtU6-*GmIPK2* sgRNA for further cloning into dephosphorylated binary vector Cas9MDC123 (Addgene plasmid ID 59184) that has *G. max* codon optimized Cas9. The cloning was confirmed by restriction analysis by using *EcoRI* and by BLAST

analysis of the vector sequences. The final Cas9/gRNA-*GmIPK2* construct was transformed into *Agrobacterium* EHA105 cells. Transformed colonies were identified by colony PCR using gRNA cassette specific primers (Table 2) that were custom synthesized based on guide RNA cassette sequence available on Addgene website (https://www.addgene.org/).

Target sequence	Primer sequence
GGTGGCCGGGCACAAGGCCAAGG	FP: 5'GATTGGTGGCCGGGCACAAGGCCA3' RP: 5'AAACTGGCCTTGTGCCCGGCCACC 3'

Table 1: Primers designed for Target 2.

S. No.	Guide RNA cassette primers	Tm (°C)
1.	Forward primer: 5'AAGCTTTCGTTGAACAACGGA3'	62.0
2.	Reverse primer: 5'AAAGCACCGACTCGGTGCCA3'	62.5

Table 2: List of guide RNA primers designed for colony PCR.

Transient expression assay of Cas9/gRNA-*GmIPK2* construct

Transient expression analysis in soybean leaves was done using AGRODATE technique. Soybean leaves of 7-week-old plants were collected and in a laminar air flow leaf discs were generated by using a cork borer. The leaf discs were rinsed with sterile water, sterilized by using 70% ethanol for 2 min; then, with 30% sodium hypochlorite for 20 min and again rinsed with sterile water. The discs were then immersed in the infiltration agro-suspension containing 10 mM 2-(N morpholino) ethane sulphonic acid sodium salt, 10 mM $MgCl_2$ (pH 5.4), 0.2M acetosyringone, 400 mg/L cysteine, 0.01% Tween-20, 0.5 mM DTT, and *Agrobacterium* cells harbouring Cas9/gRNA-*GmIPK2* with final concentration of bacterial cells optimized to OD_{600} of 0.6. After 2 h incubation, leaf discs were placed in a 20 mL disposable syringe and around 10 mL of agro suspension was drawn into it. Air gap was removed by gently depressing the plunger. The tip of the syringe was then sealed with parafilm and vacuum was created by gently pulling the plunger. Then, the syringe was shaken vigorously for 2 min, after which the plunger was released. The infiltrated leaf discs were carefully taken out, washed with sterile water, and placed on petri dishes containing MS-media with 3% sucrose, 10 mg/L sodium azide, and 0.8% agar. The petri dishes were then incubated for 4 days at 26±2°C temperature, 80% humidity, and 16:8 h photoperiod.

Genomic DNA isolation and detection of mutations

After incubation, genomic DNA was isolated from the leaf discs by using CTAB method followed by PCR amplification of the *GmIPK2* gene by using high fidelity *Pfu* polymerase to identify mutations in the target region. The PCR product was purified by using ThermoFisher Scientific Gel Extraction Kit, subjected to polyA tailing, cloned into pGEM-T vector and outsourced for sequencing. The sequencing results were analysed by using NCBI-BLAST.

Stable transformation of soybean with the Cas9/gRNA-*GmIPK2* construct

The Cas9/gRNA-*GmIPK2* construct was transformed into soybean by using cotyledonary node method. Soybean seeds were surface sterilised for 16 h in a sealed desiccator using 100ml 4% sodium hypochlorite along with 3.5mL of 12N hydrochloric acid. The sterilised seeds were placed on a germination media that contained ½ B5 media supplemented with 3% sucrose, 0.6% agar, and pH 5.8. On the 4^{th} day, explants were prepared from the soybean seedlings by removing the seed coat, separating the cotyledons, and cutting-off a major portion of the hypocotyl from the cotyledons using a scalpel. Then a small wound was made in the axillary meristematic tissue at the cotyledonary node. The half cotyledon explants were incubated for 30 min in a petri dish containing 30ml *Agrobacterium* culture harbouring the Cas9/gRNA-*GmIPK2* construct. Later, the explants were transferred to a petri dish with solid co-cultivation media containing 1/10 B5 media supplemented with 0.6% agar, 400 mg/L cysteine, 154.2 mg/L DTT, and 40 mg/L acetosyringone. The petri dishes were covered with a brown bag and kept in dark for 72 h. The explants were then transferred to a shoot induction media of pH 5.8, containing B5 media supplemented with 3% sucrose, 0.6% agar, 100 mg/L timentin, 20 0 mg/L cefotaxime, 2 mg/L glufosinate, 1.2 mg/L BAP, and 0.2 mg/L IBA. Multiple shoot induction was allowed to occur for 15 days and then glufosinate resistant plants were transferred to fresh induction medium and maintained for another 15 days. The surviving plants were further placed onto elongation media having pH 5.8 and containing B5 media supplemented with 3% sucrose, 0.6% Agar, 100 mg/L timetin, 200 mg/L cefotaxime, 2 mg/L glufosinate, and 0.75 mg/L GA_3. Leaf samples were collected from 2.5 cm long tissue culture plantlets, genomic DNA was isolated and integration of the construct was analysed by PCR amplification using gRNA cassette specific primers.

Statistical analysis

Experiments were repeated thrice and the data was represented as mean ± SE (n = 3). P value < 0.05 was considered statistically significant.

Results and Discussion

In silico analysis of the sgRNA sequences

Potential sgRNA spacer sequences (GN20GG) targeting *GmIPK2* gene were generated by using Stupar's lab CRISPR design webtool [23]. The targets were selected from the CDS (coding sequence) of the gene for effective transcriptional control. The software generated seven potential spacer sequences along with their location in the target gene and the unique restriction enzyme sites in the 15 - 19bp region of the target sequence. The sequences were further analyzed for off-target activity using Cas-OFFinder and NCBI-BLAST to short-list three sgRNA spacer sequences (Table 3), based on their uniqueness in the soybean genome and their associated high level of specificity. Activity of all sgRNAs is not the same, resulting in inconsistent Cas9 activity [24]; so, we used webtools to analyze the different parameters established for predicting sgRNA efficacy. CRISPRscan was used to check sgRNA efficacy based on sgRNA sequence composition, variable sgRNA loading, and Cas9 off-site binding. The best predicted target i.e., target-2 showed a CRISPRscan score of 59, while target-1 and target-3 scored only 39 and 43, respectively. It is known that sgRNAs having a higher probability for secondary structure formation are likely to have a better chance for target cleavage [25], probably because they facilitate the activation of Cas9 by promoting guide RNA loading onto Cas9 [26]. The tool RNAfold server was used to study the secondary structure formation (Figure 3) and minimum free energy of the sgRNAs. More negative values of MFE suggest greater stability of the secondary structure [27]. It was observed that target-2 had MFE of (-)134.30kcal/mol, while target-1 and target-3 had MFE values (-)131.30kcal/mol and (-)129.90kcal/mol, respectively. Finally, GC% of the targets was analyzed using OligoAnalyzer. Target-1, target-2, and target-3 had GC content of 65.2%, 73.9%, and 52.2% respectively. Too high and too low GC content was reported to reduce the sgRNA performance, and a range of 40% - 70% GC content was considered optimum for sgRNA activity [28]. Considering these parameters (Table 4), target-2 was most appropriate for CRISPR/Cas9-based editing of *GmIPK2* gene.

S. No.	Target/Spacer sequences	Location of the target	Restriction sites
1.	GCTCAAGATCCCGGAG-CACCAGG	3bp	HgiAI, Alw21I
2.	GGTGGCCGGGCA-CAAGGCCAAGG	24bp	StyI, EcoT14I
3.	GTGGTGTACGAGA-AGGATCTAGG	664bp	Mf1I, Xho1I

Table 3: List of target sequences along with their location and restriction enzyme sites.

Figure 3: PSecondary structures of (a) spacer sequences and (b) sgRNA sequences.

S. No.	CRISPRscan score	Mismatch number	MFE of target sequence (kcal/mol)	MFE of sgRNA (kcal/mol)	Position	GC%
1.	39	0	- 4.6	- 131.30	Exonic	65.2
2.	59	0	- 9.2	- 134.20	Exonic	73.9
3.	43	0	0	- 129.90	Exonic	52.2

Table 4: *In silico* tool-based sgRNA efficiency prediction values.

Designing and generation of Cas9/gRNA-*GmIPK2* construct

Primers for target-2 were designed with restriction enzyme (*BbsI*) sites to facilitate cloning. 'GATT' was added to the 5' end of the sense oligo and 'AAAC' to the 5' end of the anti-sense oligo (reverse complementary sequence). Equal amounts of the sense and anti-sense strands were synthesized and annealed to generate a double stranded insert which was used for ligation into *BbsI* digested pBlu/gRNA plasmid. This cloning process helps to fuse the spacer (target) sequence to the sequence for the sgRNA scaffold, ultimately generating a customizable sgRNA under the control of U6 promoter. The 5' spacer controls the Cas9 specificity while the 3' sgRNA scaffold controls sgRNA loading and Cas9 activation. The pBlu/gRNA-*GmIPK2* plasmid so obtained was restricted by using the unique restriction enzyme StyI (Figure 4a) and also sequenced commercially by using M13 primers which cover the spacer sequence as well as the 5' and 3' regions flanking the guide RNA cassette, to confirm the cloning. The final sgRNA expression cassette had a U6 polymerase III promoter, a protospacer sequence targeting *GmIPK2*, a sgRNA scaffold, and a termination sequence. The pBlu/gRNA-*GmIPK2* plasmid was digested with *EcoRI* and analyzed on 1% agarose gel (Figure 4b). The 557 bp (gRNA cassette) band was excised from the gel, purified, and subjected to alkaline phosphatase treatment to avoid self-ligation. Subsequently, it was cloned into *EcoRI* restricted and dephosphorylated Cas9 MDC123 (destination vector). Positive clones of Cas9/gRNA-*GmIPK2* were identified by colony PCR by using gRNA cassette specific primers. Then plasmid was isolated and digested with *EcoRI* to obtain a band of ~557bp size (Figure 4c). The isolated plasmid DNA was also sequenced commercially to confirm the integration of target-2 sequence. The Cas9/gRNA-*GmIPK2* construct (Figure 5a) thus generated, consisted of a 35S promoter for Cas9 expression, NOS terminator, guide RNA cassette driven by U6 promoter, a kanamycin resistance gene for bacterial selection, and bar gene as selectable marker for transgenic plant selection. This construct was transformed into *Agrobacterium* EHA-105 cells for further use.

Transient expression assay of Cas9/gRNA-*GmIPK2* construct

The recalcitrance of soybean towards transformation procedures is often dreaded by researchers due to uncertainty of the outcome. Thus, checking the efficacy of designed construct before moving onto stable transformation is considered essential for avoiding futile use of time and resources. The Cas9/gRNA-*GmIPK2* construct was transiently transformed into leaf discs of soybean (DS-9712) by using AGRODATE method. After treatment, genomic DNA was isolated from the leaf discs and transformation was confirmed by checking the integration of the Cas9/gRNA-*GmIPK2* construct by using PCR amplification of gRNA cassette (Figure 5b).

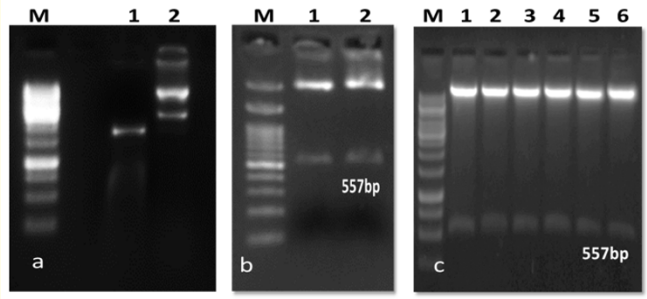

Figure 4: Binary construct development (a) Restriction analysis of pBlu/gRNA-*GmIPK2* construct with StyI (Lane M, 1 kb DNA ladder; Lane 1, Linearized pBlu/gRNA-*GmIPK2* plasmid; Lane 2, Uncut pBlu/gRNA-*GmIPK2*) (b) Restriction analysis of pBlu/gRNA-*GmIPK2* with EcoRI(Lane M, 100 bp DNA ladder; Lane 1 and 2, 557 bpgRNA cassette) (c) Restriction analysis of Cas9/gRNA-*GmIPK2* with EcoRI(Lane M, 1 kb DNA Ladder; Lane 1-6, 557bp gRNA cassette from Cas9/gRNA-*GmIPK2* constructs.

Detection of mutations in genomic DNA

Once the integration was confirmed, genomic DNA was isolated from the leaf discs, *GmIPK2* gene was amplified (Figure 5c) using a high fidelity DNA polymerase (*Pfu* polymerase), polyA-tailed and cloned into pGEM-T for sequencing to check for insertions/deletions in the gene. The sequencing results showed deletions in the target region in three of the five samples (Figure 5d). This affirms the efficacy of Cas9/gRNA-*GmIPK2* construct to cause double-stranded breaks in the target site, which resulted in deletions owing to the erroneous NHEJ repair. These deletions have potential for gene disruption by changing the reading frame of mRNA sequence.

Stable transformation of soybean with the Cas9/gRNA-*GmIPK2* construct

Once the efficacy of the construct was evident, it was used for stable transformation into soybean by cotyledonary node method (Figure 6). This method has previously been validated for 10-12% transformation efficiency [29]. The presence of bar gene in

the Cas9/gRNA-*GmIPK2* construct favoured growth of only transformed plants in shoot induction media and elongation media. Leaf samples were then collected and genomic DNA was isolated for further analysis. Integration of the construct was confirmed by PCR analysis by using gRNA-cassette specific primers. A 557bp band corresponding to the gRNA cassette ensured integration of the construct in five of the tested transgenic plants (Figure 7). In the path ahead, mutational analysis of the transformed plants can be done followed by various biochemical and phenotypic testing to ascertain plant viability and improved nutritive qualities.

Figure 5: Validation of binary construct (a) Cas9/gRNA-*GmIPK2* construct designed for developing low-phytate soybean (b) PCR amplification of gRNA cassette from leaf disc genomic DNA (Lane M, 1kb DNA ladder; Lane 1-6, 557bp gRNA cassette) (c) PCR amplification of *GmIPK2* leaf disc genomic DNA (Lane M, 1kb DNA ladder; Lane 1-5, 840bp amplicon) (d) Analysis of mutations caused by Cas9/gRNA-*GmIPK2* construct in soybean leaf discs. The target sequence of the *GmIPK2* gene is indicated in red. The deletions caused in the target region of three different test samples are indicated by blue dots.

Figure 6: Stages of Agrobacterium mediated transformation of soybean. (a) Seeds germinated on germination media (4-5 days), (b) Cotyledons excised and placed on co-cultivation medium (3 days), (c) Cotyledons transferred to shoot induction medium, (d) Regenerated shoots transferred to shoot elongation medium and depicting increase in the inter-nodal length.

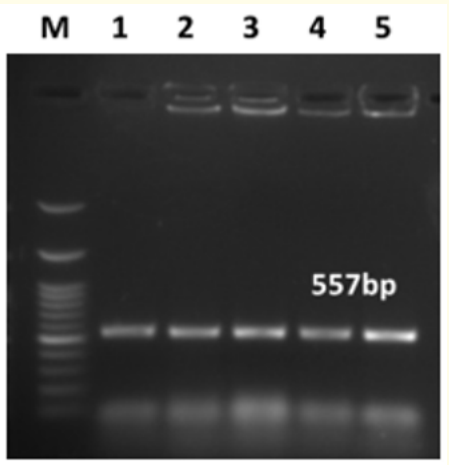

Figure 7: PCR amplification of gRNA cassette in T0 transformants by using gRNA cassette specific primers (Lane M, 100 bp DNA ladder; Lane 1-6, 557bp gRNA cassette).

Conclusion

The *in-silico* analysis of the constructs enabled selection of target sequences based on multiple molecular and thermodynamic parameters considered essential for efficient cleavage by Cas9 nuclease. By using a transient transformation method, we were able to effectively confirm the efficacy of the designed construct for causing mutations. The targeted edits obtained in this study, show the potential of the construct to cause mutations in the *GmIPK2* gene. Furthermore, soybean plants transformed with Cas9/gRNA-*GmIPK2* construct were also obtained. Downstream characterisation of these plants can yield low-phytate soybean plants. The current study is a revolutionary step towards global food and nutritional security. This study also serves as a guide for researchers seeking to effectively use CRISPR/Cas9 technology as a tool for crop improvement.

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