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CRISPR/Cas9-Mediated Knockout of Trypsin Inhibitor Genes in Soybean

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Abstract Trypsin inhibitors (TIs) in soybean are known to have antinutritional effects, reducing protein digestibility and limiting the nutritional value of soy products and animal feeds. To address this long-standing challenge, genome editing tools such as CRISPR/Cas9 have emerged as promising strategies for precisely eliminating undesirable traits such as TIs. This study explores the application of CRISPR/Cas9 to targetedly ablate trypsin inhibitor genes in soybean, specifically those encoding Kunitz and Bowman-Birk inhibitors. We discuss the biological functions and limitations of these inhibitors, outline the mechanisms and recent technical improvements of CRISPR/Cas9, and detail methods for identifying TI gene targets using transcriptomic and proteomic analyses. We also review guide RNA design, translational techniques, and gene editing validation. Functional assessments demonstrated that knockout lines exhibited reduced TI activity, improved protein digestibility, and improved nutritional status, with minimal adverse effects on agronomic traits. A case study demonstrating the successful ablation of the Kunitz trypsin inhibitor gene further demonstrates the utility of this approach. We also explore biosafety concerns, regulatory frameworks, and public perception issues surrounding genome-edited crops. Ultimately, this study highlights the transformative potential of CRISPR/Cas9 for improving the nutritional quality of soybeans and supports future efforts to integrate genome editing into breeding programs to develop high-protein, low-antinutrient varieties.

Keywords CRISPR/Cas9; Trypsin Inhibitors; Soybean; Genome Editing; Nutritional Enhancement

1 Introduction

Although soybeans are an important source of protein for humans, they are not always so "friendly" to eat. Especially for livestock that require high-protein feed or people with limited protein intake, some "extra components" in soybeans may be a problem. Anti-nutritional factors like trypsin inhibitors (TI) are not fatal in themselves, but they do affect the digestion and absorption efficiency of proteins, thereby reducing their nutritional value. The two more common types of TI-Kunitz type (KTI) and Bauman-Burke type (BBI)-are highly present in soybean seeds. They inhibit the activity of trypsin, an enzyme that is crucial for the normal digestive process in humans and animals. This kind of "inhibition" can lead to proteins not being fully utilized. Over time, it may have a negative impact on the growth and production efficiency of animals.

In fact, there were solutions in the past. Heat treatment is one of the traditional methods to remove TI, but its problems are also obvious-it consumes a lot of energy, is costly, and may even "heat" away the nutritional value of the soybeans themselves. Therefore, this approach is not suitable for large-scale, cost-sensitive food and feed industries (Wang et al., 2022).

It was not until the emergence of gene-editing tools like CRISPR/Cas9 that things began to change. Rather than "killing" TI by heating, it is better to directly "cut off" them at the genetic level. After key genes like *KTI1* and *KTI3* were knocked out, the content of inhibitors in soybeans decreased significantly, while the growth and development of the plants themselves were not affected. This approach is not only more accurate but also avoids many limitations of traditional methods (Kim et al., 2024). Even better, the mutation information generated during the gene editing process can also be used to develop molecular markers, accelerating the breeding of new soybean varieties with low TI and high nutrition (Wang et al., 2022).

This study will focus on the research progress of CRISPR/Cas9 in reducing TI content in soybeans. Besides the basic background of TI and the current editing strategies, it will also explore the potential of related technologies in commercial breeding programs, as well as the development of molecular markers and other related contents.

2 Trypsin Inhibitors in Soybean: Biological Role and Limitations

2.1 Classification and function of kunitz and Bowman-Birk trypsin inhibitors in plant defense

There is actually not just one type of trypsin inhibitor in soybeans. There are mainly two types: KTI (Kunitz type) and BBI (Bauman-Burke type) (Figure 1). KTI mainly targets trypsin, while BBI is more "omnipotent" and also has an inhibitory effect on chymotrypsin. These inhibitors are not only present in seeds but can also be found in multiple parts such as leaves, stems and roots. Almost the entire plant is equipped with this "defense system". Some studies have conducted experiments and found that after overexpressing the genes of KTI or BBI, insects like the corn borer would "keep their distance" from these plants-the number of leaves they ate was significantly reduced (Birdwell et al., 2025). This also indirectly indicates that they are indeed useful for plants in resisting insects. However, when it comes to nematodes as an enemy, these inhibitors are not as effective (Sultana et al., 2023). So, although it has a defensive function, it is not applicable to all pests and diseases.

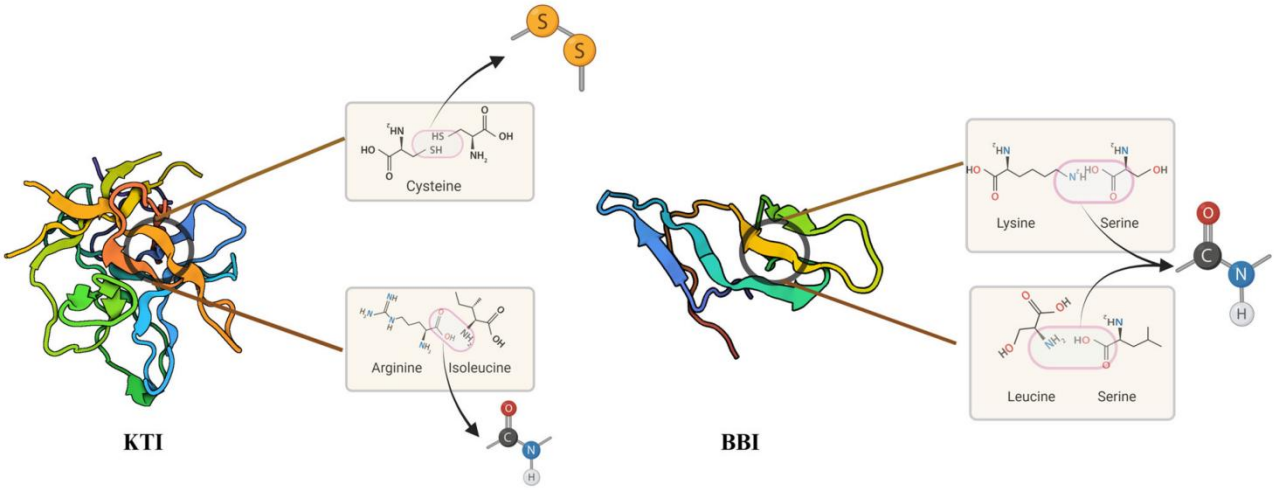

Figure 1 Two configurations of soybean trypsin inhibitors (Kunitz trypsin inhibitor (KTI) and Bowman-Birk inhibitor (BBI)) (Adopted from Luo et al., 2025)

2.2 Impact on protein digestibility and animal feed efficiency

By the way, these "protective factors" are beneficial to plants, but not good news for animals and humans. TI (trypsin inhibitor) adheres to digestive enzymes such as trypsin and chymotrypsin, disabling them. The result is a reduction in protein absorption. Especially non-ruminants, such as pigs, chickens, and humans, are more affected. If the TI content in soy products is high, the protein digestibility may even drop to around 66%. For whey protein, the content is even higher, reaching 75% (Takacs et al., 2022; Xu et al., 2022). This gap has already been able to affect the weight gain and feed conversion efficiency of animals. Another study has pointed out that a high-Ti diet can cause the pancreas of animals to become larger and its function may also be affected (Gu et al., 2023). So, from a nutritional perspective, the content of TI does indeed need to be controlled.

2.3 Challenges posed by high trypsin inhibitor content in soybean breeding and processing

The problem is not merely poor absorption after taking it. The high TI content has also caused a lot of trouble for breeding and subsequent processing. Although heat treatment can remove TI, it comes at a considerable cost-not only does it consume a lot of electricity and have a high cost, but it also destroys certain amino acids in soybeans (Luo et al., 2025). This makes it a bit difficult for processing enterprises that originally intended to produce high-quality protein products. What's more troublesome is that the TI content varies quite a lot among different soybean varieties. This genetic difference makes it particularly complicated for breeders to select varieties with low TI. Moreover, there is still a lack of molecular markers that can quickly screen for low-TI traits at present,

which also slows down the breeding pace. Ultimately, traditional methods are somewhat lagging behind, which is why there are more highly anticipated new technologies, such as genome editing-directly and precisely "turning off" genes related to TI might be the future direction.

3 CRISPR/Cas9 System

3.1 Mechanism of action: Cas9-mediated DNA double-strand breaks and repair

Initially, CRISPR/Cas9 was not discovered for gene editing. In fact, it originated from a natural "defense mechanism" used by bacteria and archaea to resist viral invasion. What truly turned it into a tool was that scientists discovered the "scissors" of this system-the Cas9 protein, as well as the sgRNA (single-guide RNA) that could guide it to its target. The working mode of this combination is actually not complicated: sgRNA will "lead" Cas9 to find the DNA region with a specific PAM sequence. Once the position is matched, Cas9 will cut that DNA and create a double-strand break. Now it all depends on how the cells respond. It may directly "suture" the wound (non-homologous terminal connection, NHEJ), but this repair method is often not very precise and is prone to losing some bases, resulting in the loss of gene function. It is also possible to use templates for repair (homologous directed repair, HDR), in which case precise modifications can be added (Jiang and Doudna, 2017; Zheng et al., 2023).

3.2 Advantages over previous gene editing tools (e.g., TALENs, ZFNs)

When it comes to gene editing, in fact, CRISPR/Cas9 is not the first "player". In the early years, there were also ZFN (zinc finger nuclease) and TALEN (transcription activator-like effector nuclease). But those who have used them all know that those methods have very high requirements for protein modification. Every time a new target is designed, a new protein has to be reconstructed, which is time-consuming and laborious. CRISPR/Cas9 is much easier in this regard. You only need to change a segment of the sgRNA sequence to make it "cut" different targets. The process is simple and the efficiency is high. This system also supports simultaneous editing of multiple genes, which is particularly convenient for the modification of complex traits. Moreover, it is less sensitive to the background of the DNA sequence or whether it is methylated than previous generations of tools, with fewer restrictions (Arora and Narula, 2017; Manghwar et al., 2019; Janik et al., 2020).

3.3 Recent improvements: multiplex editing, promoter-specific targeting, and off-target minimization

In recent years, the application of CRISPR/Cas9 has become more diverse and is no longer merely about "single-point precise strikes". For instance, it is now possible to simultaneously target multiple sites with several Sgrnas at one time to achieve multi-gene editing (Rao et al., 2022). Some people have also attempted to confine the "scissors" function of Cas9 to specific sites or time periods by using tissue-specific or promoter specific expression systems, thereby enhancing operational flexibility (Allemailem et al., 2024). The problem of missing the target was indeed worrying at first: What if the wrong place was cut? However, there have been many improvements in this area now, such as the high-fidelity Cas9 version, the optimized sgRNA design scheme, and even making Cas9 into a ribonucleoprotein complex, and even using exosomes for delivery... All kinds of means are helping to "correct" this problem (Ding et al., 2016; Horodecka and Duchler, 2021). It can be said that these advancements have truly enabled CRISPR/Cas9 to move from laboratory tools to more practical breeding and biotechnology application scenarios.

4 Targeting Trypsin Inhibitor Genes in Soybean

4.1 Identification of candidate *TI* genes through transcriptomic and proteomic profiling

The trypsin inhibitor gene (*TI*) in soybeans is not easy to recognize at a glance. To know which genes are the "main forces behind the scenes", scientists have to start from both the transcriptome and proteome levels. Through these analyses, the Kunitz (KTI) and Bowman-Birk (BBI) families gradually emerged, and they are basically the main sources of TI activity. For instance, the *KTI1* and *KTI3* genes (Glyma01g095000 and Glyma08g341500 respectively) are particularly active in seeds, and both expression data and real-time PCR indicate that they are the main "targets". This also makes them the preferred targets for gene editing (Jofuku and Goldberg, 1989; Rosso et al., 2021). The results of proteomics also confirm this point: once these two genes mutate, the content and activity of TI protein will be significantly reduced.

4.2 Design of guide RNAs for precise editing of KTI and BBI gene clusters

Editing the *TI* gene is not something that can be accomplished simply by "cutting" it. For the CRISPR/Cas9 system to function effectively, it must first have a suitable gRNA (guide RNA) to lead the way. For the KTI and BBI gene clusters, the design of grnas is usually based on their respective sequence-specific regions and expression data. Current research typically targets the open reading frames of KTI1 and KTI3 with gRNA, allowing Cas9 to create small insertions or deletions in these regions, thereby "inactivating" these genes. This operation is not done all at once but can hit multiple targets simultaneously, greatly enhancing efficiency. As for BBI, the strategy is basically similar, and loss-of-function mutants can also be obtained, ultimately achieving the goal of reducing the activity of inhibitory proteins.

4.3 Use of tissue culture and transformation systems for delivery of CRISPR constructs

Loading CRISPR tools into soybean cells is not as simple as just "tapping and importing". The method commonly used by researchers is Agrobacterium-mediated, and the objects of operation are generally the cotyledon segments or embryonic tissues of soybeans. Agrobacterium can help "send in" the CRISPR system, allowing it to be stably expressed within cells. Afterwards, through tissue culture, complete plants can be cultivated from these edited cells. Then, screening and genotype analysis are still necessary to confirm which individuals actually carry the desired mutations. For those who need to switch to breeding projects, it is necessary to identify the double-homozygous mutants without exogenous DNA again. This process, although cumbersome, has been proven effective in the editing experiments of KTI and BBI, and has greatly accelerated the development of low-TI soybean varieties.

5 Outcomes of Gene Knockout: Functional and Agronomic Evaluation

5.1 Reduction in trypsin inhibitor activity confirmed by biochemical assays

Has the gene been cut in the right place? Just imagining is not enough; one still needs to look at the data from biochemical analysis. For editing systems like CRISPR/Cas9, whether the target protein is finally handled or not usually depends on the detection of protein activity or abundance to confirm. The *FAD2* gene is a typical example. After being pruned, its fatty acid composition changed significantly, which is basically evidence that it was "knocked out" (Zhang et al., 2023). Although the current activity data for TI is not yet sufficient, it does not mean that no one has made similar attempts. Methods like RT-qPCR and protein quantification are still relatively common approaches in confirming the success of gene knockout and the decline in protein levels.

5.2 Unintended effects on plant growth, seed viability, or yield

Can plants still grow normally after one gene is cut off? This is the problem that many people are most worried about. To figure this out, researchers conducted numerous "physical examinations" on these gene-edited soybean and rice strains. Most of the results are still quite optimistic. For instance, several soybean varieties that had FAD2 knocked out grew quite normally, with no change in yield and even taller plant types. Some even demonstrated better resistance to lodging. On the rice side, after knocking out certain target genes, no significant differences were observed in pollen viability and yield (Kim et al., 2019). But then again, the absence of obvious external changes doesn't mean nothing has happened. Some potential metabolic or physiological fluctuations may require more sophisticated assessment methods to be detected (Bouche and Bouchez, 2001).

5.3 Enhanced protein digestibility and improved nutritional profile of edited soybean lines

At present, many studies focus on "visible" properties such as oleic acid content and fatty acid profile, but logically speaking, changing the target can also work. Anti-nutritional factors such as trypsin inhibitors (TI), if reduced through gene knockout, theoretically would enhance the digestibility of protein, which would be beneficial to the nutritional value of soybeans. Previous successes in oilseed traits, such as the promotion of high oleic acid soybean varieties, have already demonstrated that gene editing can indeed lead to quality improvement. On this basis, editing for TI is expected to enhance the protein utilization efficiency of soybeans, thereby providing a new breakthrough for breeding improvement.

6 Case Study

6.1 Experimental setup: cultivar selection, editing strategy, and transformation technique

When many people do soybean gene editing, their first reaction is to use Williams 82 (abbreviated as WM82). The reason is not complicated: The genomic information of this variety is relatively complete, it is widely used, and the data is also complete. So for this experiment, the WM82 was also chosen for the trial operation. The target genes are *KTI1* (Gm01g095000) and *KTI3* (Gm08g341500)-they are basically the "main force" of TI activity in soybean seeds (Figure 2). The research team designed guide RNA (gRNA) to specifically target the open reading frames of these two genes, inducing small fragment deletions or insertions, thereby rendering them "ineffective". As for how to deliver the CRISPR system into soybeans, the method remains the old one: Agrobacterium transformation + tissue culture. By sending the construct into the explant and then through tissue regeneration, the edited plant can be obtained (Wang et al., 2023).

6.2 Validation of mutations: sequencing results, inheritance, and expression analysis

It needs to be verified whether the cut is on the right spot after it is done. Researchers first confirmed through sequencing that frameshift mutations had indeed occurred in KTI1 and KTI3, with small insertions or deletions causing the loss of gene function. Protein detection further clarified the issue-the edited KTI1 protein was truncated and had lost its ability to inhibit trypsin. Interestingly, the study also identified a T1 generation strain (#5-26), which simultaneously carries homozygous mutations of kti1 and kti3, and does not carry the exogenous Cas9 gene. This indicates that mutations can be stably inherited and the expected traits can be retained without the use of genetically modified components. In addition, expression analysis also supported this result: functional KTI1 and KTI3 proteins were no longer detectable in this type of edited plants.

6.3 Results and implications: reduction in anti-nutritional activity and enhanced food/feed value

Has TI's level dropped or not? The result is crystal clear. The KTI content and trypsin inhibitory activity of the double mutant were significantly lower than those of the wild-type WM82 and the commercially available varieties. If ranked by the strength of inhibitory activity, it would roughly be: KTI1/3 double mutant < kti1 single mutant ≤ known low TI germplasm < WM82 < commercial control. The most crucial point is that although KTI1 and KTI3 were cut off, this did not affect the plant's own growth, seed development or maturation. In other words, what should grow will still grow, and what should be tied will still be tied. This gives people a lot more confidence in the practical application of these editing strains: not only is the protein digestibility higher and the nutritional value better, but also the cost of heat treatment during processing is saved. Moreover, these achievements can also provide a basis for the subsequent development of low-Ti molecular markers, indirectly accelerating the breeding process.

7 Biosafety, Regulatory, and Ethical Considerations

7.1 Regulatory landscape for genome-edited crops across major jurisdictions

For genome-edited crops, such as soybeans modified with CRISPR/Cas9, the regulation of each country cannot be generalized. In the United States, the regulatory mechanism may sound complex, but generally speaking, the Department of Agriculture (USDA), the Food and Drug Administration (FDA), and the Environmental Protection Agency (EPA) each have their own areas of responsibility. They will make judgments based on technical features and product uses instead of labeling "genetically modified" right from the start. In recent years, the United States has also been considering simplifying procedures and integrating biosafety and biosecurity for unified management (Beeckman and Rudelsheim, 2020; Cao, 2021; Li et al., 2021). Looking at the EU side from the opposite perspective, its attitude is rather strict. At present, they treat all new genomic technology products, including CRISPR, as genetically modified organisms (GMO). However, the situation is also changing. The EU is already discussing whether to distinguish between traditional genetically modified and precisely mutagenic products (Ongu et al., 2023). China also has specific regulations. The Biosecurity Law, which was introduced in 2020, provides a governance framework. However, there are still many unanswered questions regarding how to implement it and how to respond to the rapid iteration of technology (Han et al., 2020). As for some developing countries, such as certain regions in Africa, although they have established a biosecurity framework, practical

problems-like high regulatory costs and long approval cycles-often become the "roadblocks" for its promotion and application.

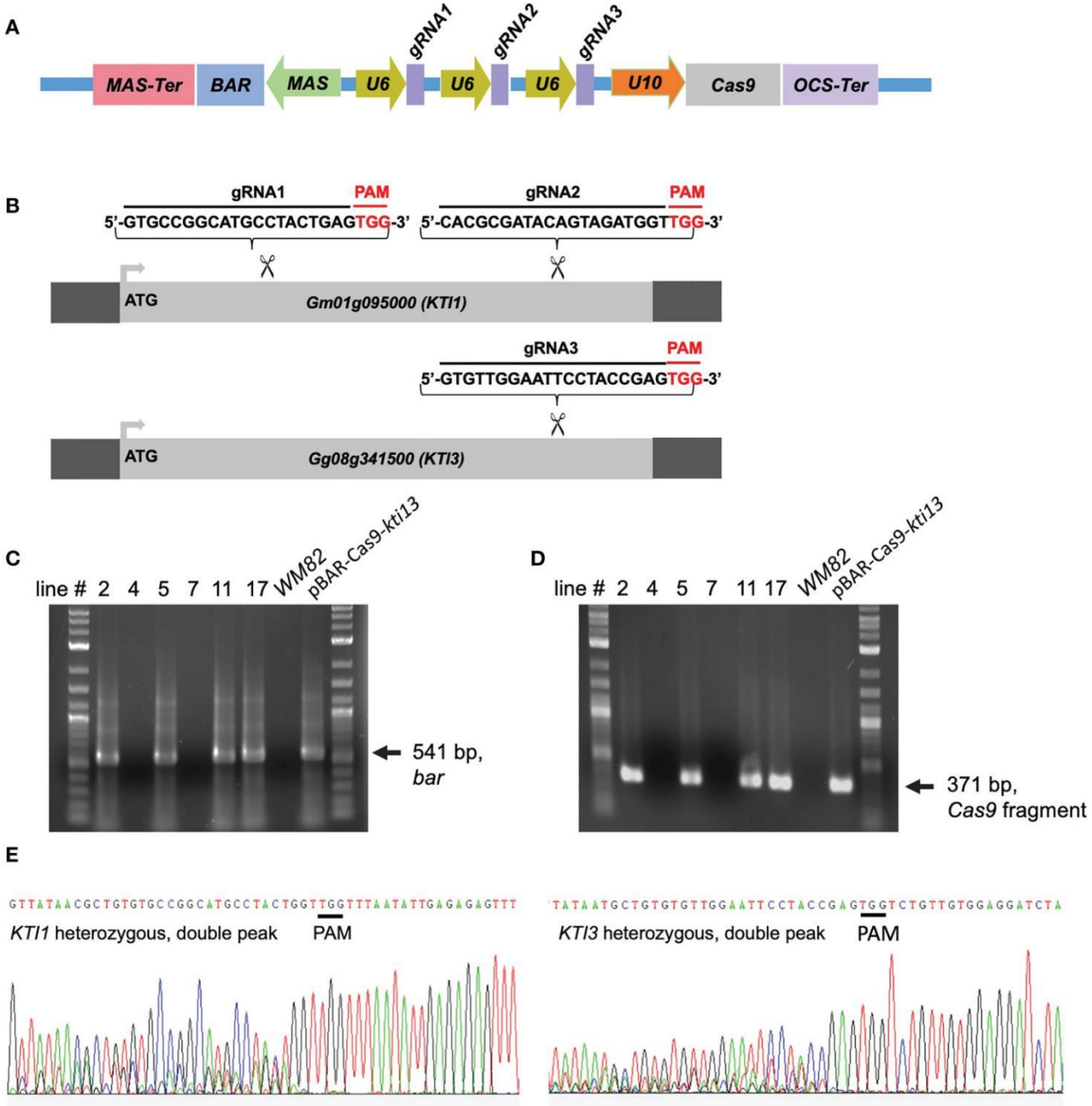

Figure 2 The scheme of binary vector used for CRISPR/Cas9 mediated gene editing on *KTI1*/*KTI3*, and transgenes and gene editing have been detected in the leaves of four T0 soybean plants. (A) The CRISPR/Cas9 construct harbors three necessary elements exhibited as below: the selection cassette consists of MAS promoter, *Bar* gene (soybean transformation selection marker), and MAS terminator; the Cas9 cassette consists of U10 promoter, Cas9 gene, and OCS terminator; three guide RNA cassettes and each of them consists of a U6 promoter, and one sgRNA. (B) The sequences of three sgRNAs is shown here. Two sgRNAs were designed, synthesized, and assembled to the plasmid to target on *KTI1*, while one sgRNA was designed, synthesized, assembled to the plasmid to target on *KTI3*. The fragments of two transgenes, (C) *Cas9* and (D) *Bar*, have both been detected in lines #2, #5, #11, and #17 by PCR, but not lines #4 and #7. The *WM82* gDNA serves as the template for negative control, while the plasmid DNA serves as the template for positive control. (E) The gene editing on *KTI1* and *KTI3* has also been observed in the leaf tissues of plants at T0 generation. The double peak sequence around the sgRNA region indicates the gene editing was ongoing but not completed (Adopted from Wang et al., 2023)

7.2 Public perception and acceptance of gene-edited soybeans with reduced anti-nutritional factors

Not everyone is willing to eat "gene-edited" soybeans, especially when the topic involves "altering DNA", public attitudes are often complex. Especially, modifications like reducing anti-nutritional factors, which seem "beneficial to health", may not necessarily win the trust of everyone. Apart from safety itself, the public's awareness of issues such as ethics and the naturalness of food can also affect acceptance. Moreover, different stakeholders have different positions, values, and understandings of risks, and these differences sometimes make policy-making more challenging (Pillai and Raybould, 2023). Blindly "popularizing science" may not be effective. A more realistic approach is to involve the public and stakeholders earlier to discuss and make decisions together, so as to reduce subsequent resistance (Asin-Garcia et al., 2023).

7.3 Strategies for transparent communication and risk-benefit analysis

Biosafety is not a matter of having it approved and then everything is fine. Transparency is the key to truly reassuring the public. According to the suggestions of international organizations, risk assessment, management measures and incident reporting all need to be systematic, open and traceable (Blacksell et al., 2023). Internal assessment by scientists alone is not enough; the participation of stakeholders must also keep up. Frankly speaking about risks and clearly stating benefits, and allowing different groups to participate in the discussion earlier, these practices are more conducive to building trust. Only when ethical aspects are also taken into account, such as who will bear potential risks and how decision-making power is allocated, can the governance of biosafety be truly "complete" (Gillum et al., 2024; Resnik, 2024).

8 Future Directions and Conclusions

Just cutting out one gene is not enough to solve all the problems. There are more than one anti-nutritional factor in soybeans. Besides trypsin inhibitors, there are also "familiar faces" such as lectins and allergic proteins. Therefore, researchers have also begun to try the "one more shot" approach, knocking out multiple problematic genes simultaneously in one generation of plants.

CRISPR/Cas9 can precisely achieve this. It is not just about cutting one by one, but can launch a "multi-pronged attack" at once-the superimposed editing of multiple genes has become feasible. This approach is significantly faster than the previous method of gradually eliminating each one, especially in accelerating the acquisition of multiple ideal trait combinations, where its advantages are even more prominent.

Of course, gene-edited materials cannot be played alone. They will eventually have to "marry" traditional breeding to enter larger-scale applications. For instance, by crossing gene-edited strains with low TI with traditional superior varieties that are drought-tolerant, high-yielding and disease-resistant, it is possible to breed new soybean varieties that are high in protein, adaptable and nutritious. This direction is actually in line with the current major trend in agricultural breeding-to make modern genetic technology an "accelerator" for traditional breeding.

Ultimately, CRISPR/Cas9 is not a universal key, but it is indeed a good tool that brings humanity closer to "precision breeding". The goals that were difficult to achieve in crop improvement in the past are now gradually becoming possible. Reducing anti-nutritional factors and allergic proteins in soybeans is just the beginning; there is still much potential to be unlocked in the future.

However, as technology advances rapidly, regulation must also keep up. Don't forget that the public's acceptance, the support of legal norms, and the sense of responsibility of the scientific community itself will all affect how far CRISPR can go. To truly enable this technology to achieve its maximum value in agriculture, it is not solely dependent on laboratory data.

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Conflict of Interest Disclosure

The author affirms that this research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

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