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Gene Editing-Assisted Development of Herbicide-Resistant Lentils

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Abstract Lentil (*Lens culinaris*) is a globally important legume crop, but its yield is increasingly constrained by weed competition and limited access to selective herbicides. This study explores how gene editing technologies, particularly the CRISPR/Cas system, can revolutionize the development of herbicide-resistant lentil varieties. We first discuss the potential mechanisms of herbicide resistance in plants, including target site resistance (TSR) and non-target site resistance (NTSR), and compare transgenic approaches with endogenous gene editing strategies. We then review advances in gene editing platforms, such as base editing and primer editing, and examine delivery systems for lentil. We focus specifically on recent advances in editing key genes, such as *ALS* (*acetolactate synthase*) and *EPSPS* (*5-enolpyruvylshikimate-3-phosphate synthase*), followed by field evaluation of gene-edited lines. We also critically analyze regulatory frameworks, biosafety concerns, and public acceptance. A case study of imazethapyr-resistant lentil breeding in Canada showcases the practical applications and achievements of gene editing in lentil breeding. This study highlights the potential of combining gene editing with modern breeding tools to broaden the herbicide resistance spectrum, enhance sustainability, and ensure the sustainability of lentil production under changing agroecological conditions.
Keywords Gene editing; Herbicide resistance; CRISPR/Cas; Lentil breeding; *ALS* gene

1 Introduction

Among the many legumes, lentils (*Lens culinaris* Medik.) may not be the "star players", but they still firmly hold their place globally due to their rich protein content and unique position in sustainable agriculture. However, when it comes to actual cultivation, there are also quite a few problems. For instance, lentils grow slowly in the early stage, have shallow root systems and relatively weak competitiveness, which makes them almost powerless against weeds-once weeds invade, the yield loss can be very serious (Balech et al., 2023). In the past, farmers mainly relied on manual weeding or crop rotation to control weeds, but these methods were labor-intensive and time-consuming, and large-scale promotion was not realistic. So, people turned to chemical herbicides. The effect was obvious, but the plant toxicity problem that followed caused harm to the lentils themselves-in some extreme cases, the yield could even drop by half. No one is willing to lose their crops for weeding.

Against this backdrop, cultivating lentil varieties that can withstand herbicides has become an unavoidable goal. This resistant variety can be used in combination with broad-spectrum herbicides, which can kill weeds without accidentally harming the lentils themselves. Naturally, it is expected to increase yields and reduce costs. However, traditional breeding methods have always been limited in this regard: there is too little natural variation, the traits are complex, and the progress is slow. Even mutant breeding or transgenic methods are often criticized-either for their low efficiency or being blocked by policies and public opinion (Singh et al., 2021). Nowadays, gene editing technology, especially CRISPR/Cas9, seems to have reached a turning point (Wang et al., 2024). It operates more precisely and efficiently, and has the potential to bypass the genetically modified label-a feature that has already shown initial success in many crops (Kuang et al., 2024). This also makes people look forward to its application on lentils.

The focus of this study is precisely to sort out the latest progress of gene editing in the development of herbicide-resistant lentils. We will elaborate from several aspects: First, we will introduce the basic situation of lentil production and its vulnerability in the use of herbicides; Then explore the genetic basis of its resistance traits;

Then focus on the practical application of technologies such as CRISPR/Cas9 in the targeted improvement of lentil traits; Finally, discuss how to incorporate these techniques into the lentil breeding system, as well as the challenges and possibilities faced. We have particularly referred to the latest achievements in lentils and related crops, attempting to present a perspective that is both realistic and forward-looking to explore how lentils can achieve more sustainable development driven by technology.

2 Herbicide Resistance Mechanisms in Plants

2.1 Target site resistance (TSR)

There are many ways for plants to develop resistance to herbicides, and among them, the most frequently occurring one is the so-called target resistance (TSR). In layman's terms, it means that herbicides can no longer find a "target". This type of resistance usually occurs when there is a "mistake" in the protein-coding gene of the target of herbicide action-not a loss of function, but a change in the appearance of the key part. If a mutation occurs at a certain site in the *acetyllactate synthase* (*ALS*) gene, the result is that the herbicides that were supposed to inhibit it fail. The same situation also occurs in genes such as *ACCase* or *EPSPS*, and their mutations can also render specific herbicides ineffective (Wei et al., 2022). But interestingly, most of these mutations are not "destructive", but rather like non-synonymous SNPS or small insertions/deletions that change the lock but do not affect the normal opening of the door. Nowadays, CRISPR/Cas9 and base editing tools are transforming these mutations from "accidental occurrence" to "targeted design", significantly enhancing breeding efficiency (Tian et al., 2018).

2.2 Non-target site resistance (NTSR)

Not all plant resistance to drugs depends on "modifying the target". Sometimes, plants even quietly get rid of herbicides before they reach their targets-this is non-target resistance (NTSR). This resistance does not take the "direct confrontation" route but rather relies on various detour methods: some make it difficult for the herbicide to be absorbed, some prevent it from entering the cell transport channels, and others directly isolate it within the plant (Gaines et al., 2020). More commonly, it is through metabolic means to "defuse the crisis", such as cytochrome P450, glutathione S-transferase and glycosyltransferase. These enzymes are like detoxification factories in plants, breaking down herbicides in advance. These mechanisms often involve the collaborative work of multiple genes, unlike TSR which can be resolved by a single "key mutation", making breeding more challenging. Even so, with the development of genomic tools and editing methods, achieving targeted regulation in the future may not be out of reach (Dong et al., 2021).

2.3 Transgene-based resistance vs. endogenous gene editing

Using foreign genes to endow plants with herbicide resistance is no longer a novelty-in the early years, the *EPSPS* or *bar* genes of bacteria were transferred into crops in this way. This approach is direct and effective, but it cannot avoid the label of genetically modified organisms. This has kept it stuck at the threshold of regulatory approval and public opinion for a long time (Hussain et al., 2021). In contrast, endogenous gene editing sounds much more low-key. It is not "borrowing genes", but making some "fine-tuning" to the existing genes of the plant itself, such as directly modifying target genes like *ALS*, *ACCase* or *EPSPS* (Figure 1) (Wang et al., 2020). Technical means include CRISPR/Cas9, base editing, and even oligonucleotide-induced mutations. Because no exogenous fragments are introduced, this method faces much less regulatory pressure and is more easily accepted by the market. Some current achievements also prove that this strategy is not only applicable to model plants, but also feasible in crops such as lentils, and the effect is stable, heritable and free of genetically modified labels.

3 Gene Editing Technologies for Lentil Improvement

3.1 CRISPR/Cas-based platforms

The popularity of CRISPR/Cas9 is no accident. Simple, precise and highly efficient-these features have enabled it to quickly gain a firm foothold in plant gene editing. In leguminous plants, even for crops with weak technical foundations like lentils, some people have begun to attempt to use CRISPR/Cas9 to specifically knock in or knock out target genes, especially to modify traits such as herbicide resistance (Ahmar et al., 2020). To be honest, there aren't many actual successful cases in the field of lentils yet. What truly restricts it is not the technical principle but the insufficiency of supporting resources, such as genomic information and stable transformation systems. But the good news is that these basic conditions are being gradually filled (Polowick and Yan, 2023). Moreover, the CRISPR family is not limited to Cas9. For instance, Cas12a excels in multi-gene editing and can target several sites at once, which is particularly useful for complex traits (Abdelrahman et al., 2021). This also shows the possibility of achieving coordinated improvement of multiple traits in lentils.

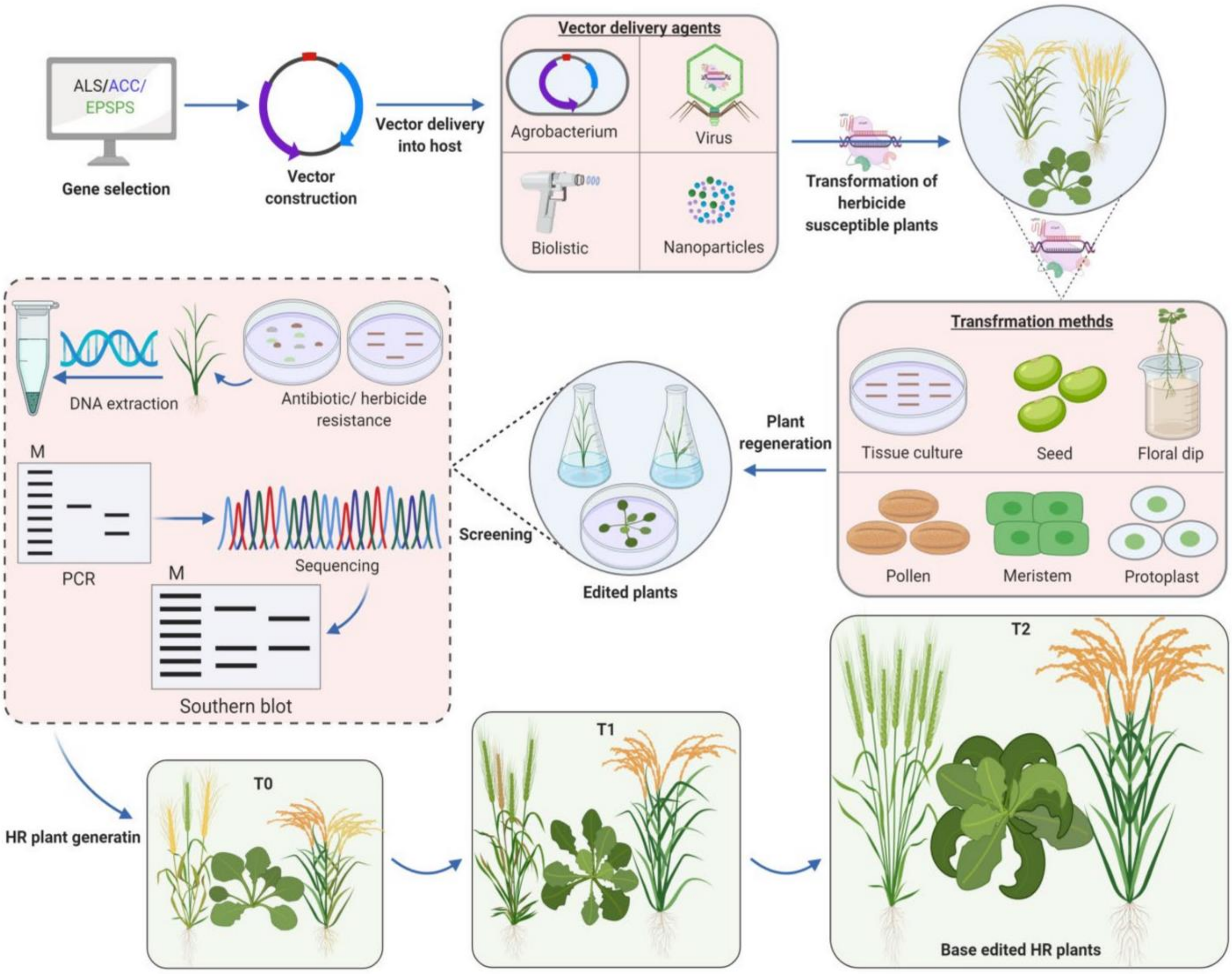

Figure 1 Use of genome editing for the development of herbicide resistance in plants. An herbicide resistant gene (*ALS*, *ACC* or *EPSPS*) is chosen and a particular target site within that gene is selected that is subsequently subjected to point mutation by base substitution. After target selection, vector construction is performed. The vector is delivered into a plant species via different methods, which is followed by plant transformation through different processes and the edited plants are regenerated. After that, the edited plants are screened for desired mutations by various methods, such as herbicide or antibiotic analysis followed by PCR, southern blotting, and sequencing. After achieving desired mutant plants (herbicide resistant plants), they are screened for particular herbicide resistance by applying the herbicide at T0, T1 or T2 generations. Typically, the base-editing generates non-transgenic (non-GM) plants (Adopted from Hussain et al., 2021)

3.2 Base and prime editing techniques

Sometimes, cutting off an entire piece of DNA is not always the optimal solution. Compared with the traditional double-strand break, the current base editing and Prime editing are like more delicate "penknives", directly rewriting individual letters without the need to create breaks. Like the base editor, by binding the Cas protein to cytidine deaminase or adenine deaminase together, C can be changed to T, or A to G-which is very convenient when making point mutations, especially when building herbicide resistance (Arora and Narula, 2017). As for Prime editing, it can achieve more complex editing, such as insertion and deletion at specific sites, and even arbitrary base replacement, with higher flexibility and a lower off-target rate (Zhu and Zhu, 2022). Although these two new tools are still in the "experimental exploration" stage on lentils, judging from the results of other crops, they have every potential to precisely improve the traits of lentils without introducing exogenous genes.

3.3 Delivery methods and transformation systems in lentils

Ultimately, no matter how advanced the editing tools are, they can only function effectively through the step of "sending in". And this is precisely the point that has always been a headache in lentil breeding. Agrobacterium-mediated method is still the most commonly used one at present. Usually, the hypocotyl or apical embryo is selected as the explant, which can increase the regeneration rate and transformation success rate. However, things did not go so smoothly. Like many leguminous plants, lentils are inherently difficult to "transform"-with low transformation efficiency and hard regeneration, which has almost become a common problem (Baloglu et al., 2022). To bypass this bottleneck, researchers are also exploring other routes, such as using gene guns to inject, using protoplasts for transient transfection, and even using nanoparticles as "couriers", attempting to complete the delivery without damaging the tissue (Ahmar et al., 2021). Although these methods are still being refined, at least the path of gene editing with broad beans is gradually moving from being "stuck" to being "open to traffic".

4 Current Progress in Herbicide-Resistant Lentil Development

4.1 Targeted editing of *ALS* and *EPSPS* genes

In the herbicide-resistant breeding of lentils, the *ALS* gene can basically be said to be the "highlight". This path has already been targeted by many studies. In the past, researchers often treated lentils with chemical mutagens such as EMS or sodium azide, and then screened out variants that showed resistance to *ALS*-suppressing herbicides (such as metochon and metochon) (Rizwan et al., 2017). For instance, in some mutant strains, the *ALS* activity has increased, but the response to herbicides has weakened. Clearly, this provides a good starting point for resistance (Shivani et al., 2022). Of course, *ALS* is not the only target; *EPSPS* are also involved, although their role is relatively weak. Interestingly, a mutation named Ala251Thr was also discovered in the *psbA* gene within chloroplasts. This change led to resistance in certain lentil strains to the PSII inhibitor metochlor, representing a novel target resistance mechanism.

4.2 Development of gene-edited lines under field conditions

Just conducting mutations in the laboratory is not enough; the lentils must be able to "withstand wind and rain". Therefore, some strains have been brought to the fields for testing and have also been compared and verified in different environments. Based on the current data, some lentils that have undergone mutagenesis or genetic modification treatment have shown stable performance. Not only do they have good resistance, but their agronomic traits have also not been compromised. For instance, ALS or psbA mutant strains with a 33-fold increase in resistance to metochlor not only performed well in greenhouses but also managed to hold their ground in real field conditions (McMurray et al., 2019). In addition, some people introduced the bar gene into lentils using Agrobacterium, and as a result, they successfully obtained plants that could survive under high-intensity herbicide pressure. This has enabled the herbicide-resistant breeding of lentils to move from a "theoretical plan" to "practical implementation".

4.3 Comparative performance with non-edited varieties

Judging from the results, the edited lentils were indeed more resilient when herbicides were applied than the common varieties. The fact that the yield has not decreased, the plants are healthy, and the entire growth process is relatively stable all make resistant varieties more attractive in practical applications. But then again, not every resistant strain is so perfect. Especially for those gene mutations that occur in chloroplasts, sometimes there will be a decrease in photosynthetic efficiency or fluctuations in yield (McMurray et al., 2021). Overall, however, the performance of some mutant strains remains impressive, even surpassing that of the original varieties, which endows them with considerable potential for subsequent breeding and commercial promotion (McMurray et al., 2018).

5 Regulatory and Biosafety Considerations

5.1 Global regulatory landscape for gene editing

The attitudes of different countries towards gene editing actually vary quite a lot. In some places, such as the United States and Argentina, only the "result" is considered-as long as the final product does not contain exogenous DNA, even if high-tech means are used, the regulatory authorities will not regard it as genetically modified organisms, which is basically the same as traditional breeding (Custers et al., 2019). But in some regions, it is not so lenient. The EU follows a "process-oriented" approach. Regardless of whether foreign fragments are introduced or not, as long as gene editing technology is used, it must be controlled in accordance with transgenic standards and undergo a complete set of strict risk assessments before being marketed (Zhang et al., 2020). Moreover, international agreements like the Cartagena Protocol on Biosafety, although providing a unified framework, have their own decisions on how to understand and implement them (Movahedi et al., 2023). Interestingly, some African and Asian countries are still "catching up". Either their systems have not yet been established, or they lack technology and manpower, and the public is not very familiar with this matter.

5.2 Off-target effects and genomic stability

Ultimately, what everyone is most worried about is still "What if something goes wrong?" No matter how precise the CRISPR/Cas technology is, it cannot guarantee that the operation will only be performed at the predetermined position each time. Off-target effects may quietly modify other genes and sometimes bring about some unexpected phenotypes, such as triggering allergic reactions or generating certain potential toxicities (El-Mounadi et al., 2020). However, the good news is that technology has indeed advanced in recent years. For instance, high-fidelity Cas proteins and optimized guide RNA designs have significantly reduced risks, but they are still far from being "completely resolved" (Han et al., 2020). Regulatory authorities are now paying increasing attention to this aspect, requiring developers to provide detailed molecular data, conduct off-target detection specifically, and prove that there are no redundant transgenic fragments in the plant (Eckerstorfer et al., 2019). Moreover, it's not over once they are put on the shelves. Continuous monitoring is still necessary in the future. Any issues should be made public and clearly stated. Only in this way can we ensure that these crops are stable and safe at the genetic level (Lema, 2021).

5.3 Public perception and market acceptance

Not everyone can accept "edited food" at once. How the public views it largely depends on whether they can understand the underlying scientific principles and whether the regulation is transparent. In some countries that treat gene editing and traditional breeding equally, people tend to be less resistant, especially when the product itself does not contain genetically modified components (Lema, 2019). However, we cannot ignore those voices that still have concerns-worries about ethics, potential risks, and uncontrollable consequences, especially in areas with strict regulations on genetically modified organisms, which are quite common (Kalidasan and Das, 2021). If the market is to better accept these new products, relying solely on the technology itself is not enough. How to communicate, how to write labels, and whether stakeholders can participate all directly affect the future fate of gene-edited crops such as lentils (Rabuma et al., 2024).

6 Case Study: Development of Imazethapyr-Resistant Lentils in Canada

6.1 Project background and objectives

In Canada, when growing lentils, one is not afraid of drought or pests, but is most afraid of weeds. Once not well controlled, a decline in output will be obvious to the naked eye. Lentils themselves are not very competitive. Once weeds grow wildly, they simply cannot be suppressed. Herbicides like imidazolicotinic acid, although broad-spectrum and highly effective, have a problem-they are also "tough" on common lentils. Even the recommended doses may lead to reduced crop yields (Wall and Mcmullan, 1994). To solve this difficult problem, the breeding team in Canada decided to change their approach, with a clear goal: to develop a resistant lactil that can withstand the later application of imidazolicin, making weeding in the fields more flexible and effective, and the cultivation more stable.

6.2 Gene editing process and validation

Where does this resistance come from? The key still lies in the *ALS* gene. As the target of imidazolicotinic acid, as long as this gene becomes "able to see through the moves", it can withstand the attack of herbicides. In the past, people mostly relied on traditional mutagenesis and screening, which had some effects, but the accuracy was not high enough. Now, researchers in Canada have also begun to test the water with CRISPR/Cas9, attempting to perform "surgery" on *ALS*, introducing precise mutations, and pursuing more stable and heritable resistance (Figure 2). During the process, not only the genetic data was examined, but also field experiments were conducted: could the edited plants survive after being sprayed with pesticides? How do you look? Will there be a harvest in the end? At the same time, molecular testing was also carried out to confirm that the mutation did occur at the target site (Wall, 1996). Only when both ends are passed can it be truly considered a success.

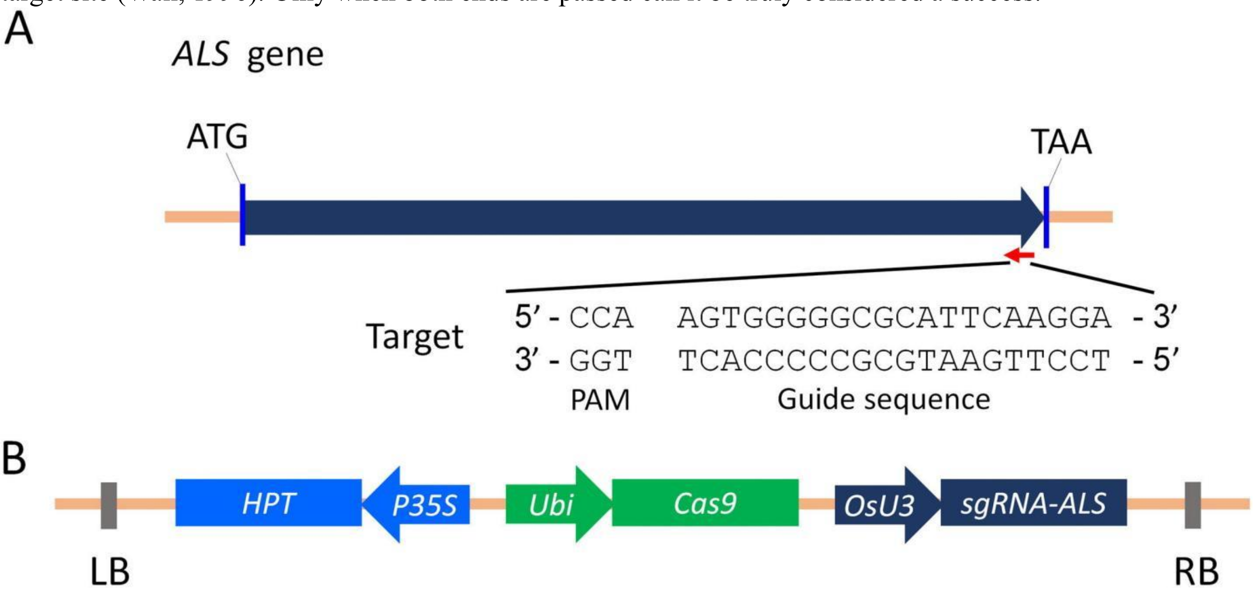

Figure 2 Schematic diagram of the targeted sequence in *OsALS* and CRISRP/Cas9-ALS vector (Adopted from Wang et al., 2020)
Image caption: (A) A schematic description of the *OsALS* gene. The target site is located at the 3′ end of *OsALS* and indicated by a yellow arrowhead antisense to *OsALS*. The sgRNA and protospacer-adjacent motif (PAM) together with their target sequences are shown. (B) Linearized CRISPR/Cas9-ALS construct showing genes encoding hygromycin B phosphotransferase, Cas9 protein, and the sgRNA targeting *OsALS* (Adopted from Wang et al., 2020)

6.3 Impact and future applications

After the emergence of resistant lentils, field management has become less tense. Originally, it was necessary to rush to spray pesticides before the weeds grew. Now, it's better to wait a little longer-to act when the crops and weeds start competing for territory. The effect will be better and the yield can be increased by about 30% (Fedoruk and Shirtliffe, 2011). This resistance strategy not only makes the timing of herbicide application more flexible but also avoids early damage to seedlings. Overall, it is more in line with the concept of sustainable agriculture. For now, this case in Canada serves as a reminder to other countries that it is not necessary to rely on genetic modification. By making good use of gene editing, lentils can also be "armed". In the future, in addition to continuing to build up the resistance to multiple herbicides, some agronomic traits can also be improved incidentally, bringing us one step closer to practical application (Shivani et al., 2023).

7 Future Prospects and Research Directions for Herbicide-Resistant Lentils

7.1 Expansion to other herbicide classes

At present, most of the research on the herbicide resistance of lentils focuses on ALS and PSII inhibitors, with an emphasis on the two types of herbicides, imidazolicotinic acid and metochlor. But one should not just focus on these two. Herbicides with different mechanisms of action, such as oxyfluoxane, fluoxazuron, triazuron and methomylimate, have long been widely used in other crops. In fact, some screening experiments and mutagenesis studies have found that there are indeed genotypes in lentils that can tolerate these herbicides. This indicates that in the future, it is possible to make lentils more composed when facing various weeds by superimposing multiple resistance genes, and it will also help reduce the probability of weeds "becoming smarter", delaying the emergence of drug-resistant weeds.

7.2 Integration with marker-assisted and genomic selection

Gene editing is not a solitary battle. When used in combination with tools such as marker-assisted selection (MAS) and genomic selection (GS), it can significantly accelerate the breeding speed of herbicide-resistant varieties. Recent genome-wide association studies (GWAS) have identified some SNPS and QTL loci related to herbicide tolerance (Balech et al., 2024; Cao et al., 2024). These markers are like "signal lights", which can help breeders pick out potential players from a large pile of materials. The addition of gene editing can directly modify the target site, achieving precise "patching" (Roy et al., 2023). Even better, this method can also simultaneously optimize other traits, such as increasing production and enhancing stress resistance, achieving multiple benefits at once.

7.3 Addressing climate and sustainability challenges

Things can't be settled once and for all. Although the herbicide-resistant varieties of lentils seem to have a promising future, their performance varies significantly under different climatic conditions. Multiple environmental tests have shown that some varieties grow well in humid environments but not so well in arid areas. The reverse is also true (Balech et al., 2022). Therefore, in the future, breeding should not only focus on "drug resistance", but also take into account "adaptability". Especially in the context of increasingly unstable global climate, developing varieties that can grow stably and maintain stable yields in various complex environments is the key. Moreover, if herbicide resistance can be combined with traits such as drought tolerance, heat tolerance and disease resistance, it might truly support a sustainable and intensive production system for lentils.

8 Concluding Remarks

The progress in lentil breeding, especially those in recent years, cannot be attributed solely to the technology itself. Indeed, tools like CRISPR/Cas offer us the opportunity to precisely manipulate and modify those key genes, thereby enhancing lentils' tolerance to herbicides, their disease resistance, and even improving the stability of their yields. But the real breakthrough was brought about by the "superposition" of a series of technologies: such as the establishment of high-density genetic maps, the discovery of linkage markers, and the wide application of sequencing technology. All these have significantly accelerated the improvement process and made it possible to develop varieties that are more adaptable to climate change.

Of course, relying solely on technology is not enough. For these resistant varieties to truly enter the fields and farmlands, it still depends on supporting policies, responsibility mechanisms and international cooperation to provide a safety net. For instance, standardized genetic resource databases, cross-border sharing mechanisms, and even public communication strategies are all indispensable components. Without these supports, even if the laboratory achievements are excellent, it will be difficult to go far. A policy environment for fair access to new technologies is even more crucial for researchers and farmers in developing countries (not just a matter of biosecurity).

In the future, lentil breeding may not rely solely on a single tool. What it requires is a "team battle"-gene editing, genome selection, and genetic diversity from wild and local breeds, all working together. Only in this way can there be hope to cultivate new varieties that are truly suitable for different agricultural areas and are both high-yielding and stress-resistant. To make all this come true, relying on just one or two research teams is far from enough. Continuous investment, accumulation of capabilities, and cross-border coordination and collaboration are the true cornerstones that can support the future of Bian Dou.

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Conflict of Interest Disclosure

The authors affirm 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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