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CRISPR/Cas9 Applications in *Ganoderma lucidum* Breeding for Enhanced Bioactive Compound Production

Shiying Yu ✉

Biotechnology Research Center, Cuixi Academy of Biotechnology, Zhuji, 311800, China

✉ Corresponding author: shiying.yu@cuixi.orgGenomics and Applied Biology, 2025, Vol.16, No.2 doi: [10.5376/gab.2025.16.0010](https://doi.org/10.5376/gab.2025.16.0010)

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Abstract This study sets out to explore the potential of CRISPR/Cas9 gene-editing technology in improving *Ganoderma lucidum*, with a particular focus on whether it can truly help boost the yield of active ingredients. The article begins with a brief overview of the fungus's main bioactive compounds-polysaccharides and triterpenoids-and their medicinal value. But here's the catch: traditional breeding methods, while useful in the past, appear to have hit a bottleneck when it comes to further improving the efficiency of these compounds' synthesis. Against this backdrop, attention has naturally shifted to CRISPR/Cas9. The paper explains the system's basic principles and advantages, then illustrates them with practical examples from fungal genetic studies. Notably, the technology has already delivered promising results in editing key genes (such as *cyp5150l8* and *cyp505d13*) and in optimizing metabolic pathways. At the same time, the authors stress that if homologous recombination efficiency could be improved-or if newer methods like ribonucleoprotein (RNP) complex delivery were applied-the accuracy and overall efficiency of gene editing could be pushed even further. Finally, the article steps back to consider the bigger picture: CRISPR/Cas9 is not just another piece of lab equipment. It may well become a powerful tool for targeted breeding of active ingredients in *Ganoderma lucidum*, while also fueling the development of new medicines and functional foods. Looking ahead, it even holds the promise of playing a pivotal role in the broader industrialization of fungal biotechnology.

Keywords CRISPR/Cas9; *Ganoderma lucidum*; Gene editing; Ganoderic acids; Bioactive compounds; Molecular breeding

1 Introduction

Ganoderma lucidum, commonly known as "Lingzhi" in Chinese, is a kind of fungus used in medicine and has been used in traditional medicine for more than two thousand years. It is well-known for its health benefits. People consume it mostly because they think it can prolong life, enhance the immune system, and reduce the risk of diseases such as cancer and heart disease (Wachtel-galor et al., 2004; Lu et al., 2020; Sheikha, 2022). *G. lucidum* contains some bioactive compounds such as polysaccharides and triterpenoids. These compounds have diverse biological activities, including antioxidant, anti-tumor, anti-inflammatory, antiviral and immunomodulatory effects. Therefore, they have been widely studied by scientists (Soccol et al., 2016; Lu et al., 2020).

G. lucidum contains many bioactive compounds, including polysaccharides (GLPs), triterpenoids, sterols, etc. These bioactive compounds are of great use in medicine. Among them, GLPs are particularly abundant in *G. lucidum* cells, and GLPs have excellent antioxidant, anti-tumor, anti-inflammatory and immunomodulatory functions (Lu et al., 2020). Furthermore, triterpenoids extracted from *G. lucidum* spores are also considered beneficial to the nerves, can resist aging, and protect cells (Soccol et al., 2016). With the deepening of research, the application value of these active ingredients in the fields of pharmaceuticals, health products, cosmetics, etc. is also receiving increasing attention (Sheikha, 2022).

The core strength of CRISPR/Cas9 lies in its ability to make highly precise modifications to DNA sequences. That's why it is so often described as efficient, simple, and versatile-with some even going so far as to call it a "revolutionary" genome editing tool. Over the past few years, its applications have expanded widely across many areas of biology. Still, compared with its relatively mature use in plants and animals, work on applying

CRISPR/Cas9 to fungal genetic improvement is only just beginning to take shape. Yet this hasn't stopped it from showing remarkable promise. Take *Ganoderma lucidum* breeding as an example. Researchers have long wrestled with the same challenge: how to further boost the yield of its active compounds. Traditional approaches have offered only modest gains. CRISPR/Cas9, by contrast, seems to open a more direct route forward. By pinpointing and editing genes closely tied to the synthesis of these active ingredients, scientists not only stand a good chance of raising compound yields significantly, but may also enhance their pharmacological effects along the way. Put differently, the medicinal value and commercial outlook of *Ganoderma lucidum* could be heading toward a new breakthrough-thanks in no small part to this technology.

The starting point of this research is actually quite straightforward: we want to identify which genes are truly “in charge” of synthesizing the active ingredients in *Ganoderma lucidum*. Once those genes are pinned down, the idea is to see whether CRISPR/Cas9 technology can be used to modify them-perhaps boosting the yield of these valuable compounds in the process. To do this, we plan to move forward in three stages. First, we need to establish a CRISPR editing method that really works for *Ganoderma lucidum*-because different species come with different quirks, and simply copying what has been done in other fungi rarely succeeds. Second, we'll focus on identifying and verifying the so-called “key target” genes, confirming how exactly they contribute to active ingredient synthesis. And finally, we'll put the system to the test: once these genes are modified, does the yield of active ingredients actually increase? Or, perhaps more intriguingly, do their biological effects change in any meaningful way? If all goes well, the outcomes of this work could do more than just advance fungal biotechnology. They may also open new doors for developing medicines and health foods based on *Ganoderma lucidum*. Put differently, this research could represent a key step in shifting *Ganoderma lucidum* from being seen purely as a “traditional medicinal material” to becoming part of a “modern bioindustry.”

2 *Ganoderma lucidum*: Bioactive Compound Synthesis

2.1 Overview of key bioactive compounds

Ganoderma lucidum is a traditional medicinal fungus. People are well-known to it largely because it contains a variety of bioactive compounds. Among them, the most closely watched are polysaccharides and triterpenoids - many studies have found that their role in promoting health is quite prominent. In addition, *Ganoderma lucidum* actually contains many other components, such as sterols (like ergosterol), proteins, nucleosides, fatty acids and some trace elements. These substances may not be as well-known as polysaccharides and triterpenoids, but they still add a lot of color to the medicinal value of *Ganoderma lucidum*.

Polysaccharides (GLPs) in *G. lucidum*, especially β -D-glucan, have a relatively high content and are the main polysaccharide components in *G. lucidum*. They can be extracted from *G. lucidum* spore powder or fruiting bodies. GLPs have diverse and complex structures (including the diversity of molecular weight, tertiary structure, side chains, substituents and monosaccharide composition), and are bioactive macromolecules with molecular weights ranging from several thousand to several million Da (Lu et al., 2020). Another key type of compound in *G. lucidum* - triterpenoids - includes ganoderic acid, ganoderic acid, ganoderol, ganoderiol and lucidenic acids. These compounds give *G. lucidum* a unique bitter taste and have attracted attention due to its diverse pharmacological properties (Ahmad et al., 2021).

2.2 Role of these compounds in health and medicine

The bioactive components contained in *Ganoderma lucidum* have always attracted much attention, mainly because they have demonstrated a considerable variety of medicinal potential. It is particularly worth mentioning *Ganoderma lucidum* polysaccharides (GLPs). Relevant studies have found that they not only exert antioxidant effects but also show positive effects in anti-tumor, anti-inflammation, anti-virus, anti-diabetes and immune regulation. Of course, most of these functions are still at the research level at present. But precisely because of this multi-faceted nature, GLPs are considered to have the potential to become an important candidate for the development of new drugs (Lu et al., 2020).

Triterpenoids also have significant value. For instance, ganoderic acid can inhibit the growth of breast cancer cells, and ganoderic acid A can have anti-inflammatory and anti-apoptotic effects. Studies have shown that such

compounds exhibit good effects in anti-inflammation, anti-cancer, liver protection and lipid regulation, and may have therapeutic potential for various diseases such as diabetes, neurodegenerative diseases and cardiovascular diseases. However, it should be pointed out that most of the current related research is still mainly in the laboratory, and more clinical trials are still needed to verify its actual efficacy and safety for humans (Ahmad et al., 2021).

2.3 Biosynthetic pathways and genetic regulation

The active ingredients in *Ganoderma lucidum* (such as polysaccharides) do not "pop up" out of thin air, but are gradually synthesized through a complex set of metabolic processes in the body, and all of this is inseparable from the precise regulation of genes. Take polysaccharides for example. Their synthesis relies on the action of a series of glycosyltransferases. These enzymes are somewhat like "assemblers" working on an assembly line, capable of specifically catalyzing the formation of glycosidic bonds, piecing together activated monosaccharide substrates (such as UDP-glucose) bit by bit, and ultimately building polysaccharide chains with specific spatial structures. However, things are not that simple. The genes themselves responsible for creating these "workers" are also subject to the regulation of transcription factors and signaling pathways. In other words, changes in environmental conditions and even the requirements of *Ganoderma lucidum* at different growth stages can affect the expression levels of these genes, thereby indirectly determining the synthesis efficiency and results of polysaccharides (Lu et al., 2020).

The triterpenoids in *G. lucidum* are mainly synthesized through the mevalvalic acid (MVA) pathway. This pathway uses acetyl-coA as the starting material and undergoes multiple enzymatic reactions to generate lanosterol. The latter then undergoes oxidation, cyclization and other modifications to form a variety of triterpenoid structures. This synthesis process is precisely regulated by genes. Under the action of regulatory proteins such as transcription factors, related genes can respond to environmental and developmental signals, thereby controlling the synthesis amount and types of triterpenoids (Ahmad et al., 2021).

3 Current Breeding Techniques in *Ganoderma lucidum*

3.1 Traditional methods of strain improvement

In the traditional breeding of *Ganoderma lucidum*, improvement has mostly leaned on rather old-fashioned tools like physical mutagenesis and artificial selection. Among these, ultraviolet (UV) irradiation is probably the most widely used. A good example is the strain "UV119," which a research team developed through UV treatment. Compared with its parental strains, UV119 stood out not only for producing much higher yields of spore powder, but also for showing stronger resistance to microbial infection (Tang et al., 2023) (Figure 1). That said, the story is not entirely rosy. While such methods can indeed deliver noticeable gains in yield and resistance, they come at the cost of an exhausting process. Researchers often have to sift painstakingly through countless mutant strains just to find a few that meet the breeding goals. Put differently, achieving real progress in this way is less a matter of a single "lucky hit" than a drawn-out effort of trial and error.

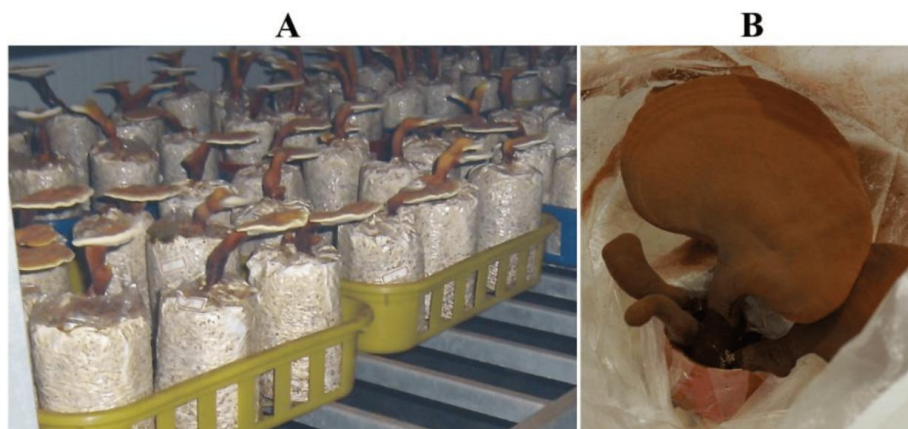


Figure 1 Fruiting of the 1st, 5th, 10th and 15th generations of the mutagenic strain UV119. (A): Fruiting situation of different generations of UV119; (B): collection of spores powder (Adopted from Tang et al., 2023)

3.2 Limitations of conventional breeding techniques in enhancing bioactive compound production

Although traditional breeding methods have achieved some results in the improvement of *Ganoderma lucidum*, they have not been very effective in increasing the yield of bioactive compounds. Take UV mutagenesis as an example. It can indeed create new variations, but such changes are often highly random - as a result, a large number of mutations are neither predictable nor beneficial to the target traits, and may even have negative impacts. As a result, researchers have to invest a great deal of time and effort in screening and validation in order to sift out a few valuable mutant strains. The entire process is not only inefficient but also quite time-consuming (Tang et al., 2023). More importantly, such traditional methods are difficult to achieve "targeted treatment" at the genetic level. However, if one wants to truly optimize the synthesis pathway of active ingredients in *Ganoderma lucidum* in a targeted manner, precise gene editing is almost indispensable. It is precisely because of the lack of such efficient targeted genetic tools that the improvement of specific traits and efficient breeding of *Ganoderma lucidum* have been greatly limited for a long time (Liu et al., 2020; Tu et al., 2021).

4 CRISPR/Cas9 Technology: Principles and Mechanism

4.1 Explanation of CRISPR/Cas9 genome editing

The CRISPR/Cas9 technology was not originally "designed" for humans; it actually originated from an immune defense mechanism of bacteria. Surprisingly, this system, which was originally designed to defend against virus invasions, has now been developed into a powerful tool capable of efficiently and precisely editing genomes. Its core structure is not complicated and mainly consists of two parts: Cas9 nuclease and single-stranded guide RNA (sgRNA). Among them, sgRNA can be flexibly designed based on the target DNA sequence, functioning as a "navigation map", responsible for precisely delivering Cas9 to specific sites in the genome. Next, Cas9 will act like "scissors" to cut the DNA double strand at the target site, creating a double-strand break (DSB). However, the cell itself will not stand by and do nothing. It will activate the repair mechanism - possibly through non-homologous end joining (NHEJ), or through homologous directed repair (HDR) to fill the gap (Ran et al., 2013; Sander and Joung, 2014; Bortesi and Fischer, 2015). It is precisely by taking advantage of this principle of "cutting first and then repairing" that researchers have been able to carry out various genetic operations, such as gene knockout, insertion of exogenous genes, and even precise point mutations. Therefore, CRISPR/Cas9 has gradually become an indispensable key technology in current gene function research and breeding improvement (Wang et al., 2016; Tavakoli et al., 2021).

4.2 Advantages of using CRISPR/Cas9 in fungal research

The CRISPR/Cas9 technology has demonstrated significant advantages in fungal research, especially in improving *G. lucidum* through gene editing and increasing the yield of its bioactive compounds. This system features high accuracy and high efficiency, and is capable of achieving targeted editing of specific genes, providing a key means for analyzing gene functions and metabolic regulatory mechanisms (Bao et al., 2019; Li et al., 2021). Compared with traditional gene editing methods, CRISPR/Cas9 is more convenient to operate and helps significantly shorten the research and development cycle and reduce costs (Belhaj et al., 2015). This technology can rapidly induce targeted mutations and significantly accelerate the breeding process, which plays an important promoting role in the selection and breeding of new germplasm of *G. lucidum* with high-yield polysaccharides, triterpenoids and other bioactive compounds (Barrangou and Doudna, 2016; Chen et al., 2019).

4.3 Comparison with other gene-editing technologies

The CRISPR/Cas9 system has quickly demonstrated unique advantages in the field of gene editing due to its simplicity of operation and diverse functions. Compared with the early zinc finger nucleases (ZFNs) or transcription activator-like effector nucleases (TALENs), its threshold is much lower. Those technologies require redesigning complex protein structures for each target, but CRISPR/Cas9 is different. It only needs to replace one sgRNA to recognize new DNA sequences. This makes it not only easier to handle, but also significantly reduces the cost (Sander and Joung, 2014; Bortesi and Fischer, 2015). What is even more remarkable is that the application scope of CRISPR/Cas9 goes far beyond "cutting" genes. It can also be extended to directions such as gene expression regulation, epigenetic modification, and even genomic imaging - which are often difficult to achieve in traditional nuclease techniques (Wang et al., 2016; Li et al., 2021). Of course, this system is not perfect

either. It may still bring about off-target effects and produce some unexpected editing results. However, researchers have proposed various improvement ideas, such as optimizing the design of sgRNA or adopting high-fidelity Cas9 variants, thereby alleviating this problem to a certain extent (Ran et al., 2013; Tavakoli et al., 2021). In other words, although CRISPR/Cas9 still has limitations, with its high efficiency and wide applicability, it still firmly ranks among the "preferred tools" in fungal genetic improvement and even broader life science research (Barrangou and Doudna, 2016; Bao et al., 2019).

5 CRISPR/Cas9 Applications in Fungal Systems

5.1 Overview of successful applications of CRISPR/Cas9 in Fungi

The arrival of CRISPR/Cas9 technology can, without much exaggeration, be seen as a real turning point in fungal genetics. Suddenly, researchers found themselves able to manipulate genomes with a level of precision and efficiency that used to feel almost out of reach. And today, its footprint is everywhere: whether you look at basidiomycetes or filamentous fungi, CRISPR/Cas9 has already become part of the basic toolkit. Take *Ganoderma lucidum* as an example. In one study, knocking out genes involved in triterpene synthesis led to dramatic shifts in metabolite composition (Wang et al., 2019; Tan et al., 2023). A similar strategy has been applied to edible fungi like *Agaricus bisporus* and *Pleurotus* spp., where gene editing has been used to enhance not only nutritional value but also potential medicinal properties (Zhang et al., 2023). Filamentous fungi tell a related, though slightly different, story. Here, CRISPR/Cas9 has proved useful not just for untangling complicated metabolic processes but also for speeding up the fine-tuning of synthetic pathways-lovastatin and betaine being two of the best-known examples (Jiang et al., 2021). All in all, this technology keeps pushing the boundaries of fungal research. From probing fundamental mechanisms to paving the way for real-world applications, CRISPR/Cas9 has opened possibilities that, just a decade ago, would have seemed more like speculation than practice.

5.2 Case studies of gene editing in other medicinal Fungi

In fact, researchers have been experimenting with CRISPR/Cas9 in medicinal fungi for quite some time now, and the outcomes are often striking. Take *Cordyceps militaris*, for example. Known for its anti-inflammatory and anti-tumor effects, it has long drawn scientific interest. By using CRISPR/Cas9 to tweak the genes tied to cordycepin synthesis, scientists managed to rewire a key metabolic process. A similar story unfolds in *Schizophyllum commune*. Here, gene editing was used to boost polysaccharide production-and as it happens, those polysaccharides play an important role in immune regulation (Zhang et al., 2023). *Aspergillus* spp. offers yet another angle. Instead of focusing on single genes, researchers applied CRISPR/Cas9 to fine-tune entire gene clusters related to secondary metabolite synthesis. The payoff was clear: its medicinal potential grew even stronger (Tong et al., 2019). Put differently, CRISPR/Cas9 is quietly reshaping how we think about improving medicinal fungi. It's not just about producing more-it's also about making them work better, opening the door to functions and applications that were once difficult to reach.

5.3 Insights into potential challenges in fungal genome editing

Although CRISPR/Cas9 technology has shown considerable potential in fungal research, when it comes to practical application, it still encounters some unavoidable technical bottlenecks. First of all, fungi like *Ganoderma lucidum* often have an old problem: the efficiency of homologous recombination (HR) is generally low, while non-homologous terminal connection (NHEJ) dominates. This means that it is not easy to achieve precise gene insertion or replacement (Figure 2) (Tu et al., 2021). Secondly, the off-target effect is also a headache. It may bring about some unexpected mutations. Fortunately, this problem is not completely unsolvable - by choosing the high-fidelity Cas9 variant and combining it with the optimized design of sgRNA, it can be alleviated to a certain extent (Ouedraogo and Tsang, 2020). In addition, how to efficiently deliver the components of CRISPR/Cas9 into fungal cells is also a key challenge that must be overcome in the research. Different fungi vary greatly and often require specialized optimization of transformation methods and vector systems (Schuster and Kahmann, 2019; Song et al., 2019). In other words, although the technical framework is relatively mature, there are still many details that need to be refined in specific operations.

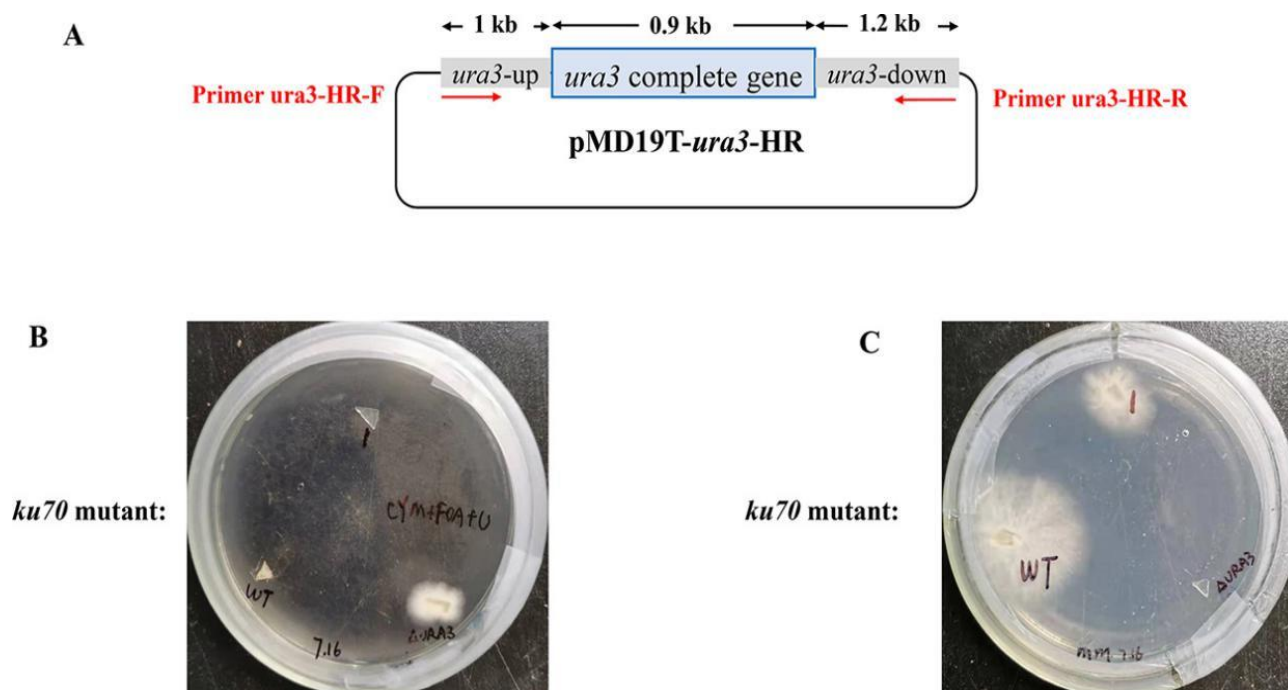


Figure 2 (A) Donor plasmid pMD19T-*ura3*-HR used for transformation of the *ura3*-*ku70* mutant. (B) Subculture of the *ku70* mutant (1) obtained, the wild-type (WT) strain, and the *ura3*-deficient strain (Δ *ura3*) on an MM plate containing 400 mg/liter 5-FOA. (C) Subculture of the *ku70* mutant obtained, the wild-type strain, and the *ura3*-deficient strain on an MM plate without uridine (Adopted from Tu et al., 2021)

6 Target Genes for Enhanced Bioactive Compound Production

6.1 Identification of key genes involved in bioactive compound synthesis

In fact, the genome of *Ganoderma lucidum* hides an unexpectedly large collection of genes linked to secondary metabolism. Among them, the cytochrome P450 monooxygenase (CYP) family is especially worth noting—they often take center stage in the synthesis of triterpenoids such as ganoderic acids (GAs) (Chen et al., 2012; Wang et al., 2019). Researchers have already identified several core genes that directly step into this process. For instance, *cyp5150l8* helps convert lanosterol into ganoderic acid, while *cyp505d13* plays a major role in producing sterol-type triterpenoids (Wang et al., 2019). You could think of these genes as interlocking gears: each one small on its own, but together driving the larger metabolic machine forward. Polysaccharides, however, follow a somewhat different script. Their synthesis isn't controlled by a single "master switch." Instead, it relies on the teamwork of many enzymes—glycosyltransferases and glycoside hydrolases among them—working in a carefully coordinated manner (Wu et al., 2022). Put simply, while the pathways that give rise to different active ingredients in *Ganoderma lucidum* each have their own logic, they all share one thing in common: a subtle, tightly regulated genetic choreography operating quietly in the background.

6.2 Potential CRISPR/Cas9 targets for improving compound yield

The introduction of CRISPR/Cas9 technology has provided a brand-new tool for the genetic improvement experiments of *Ganoderma lucidum* and made it more possible to increase the yield of bioactive compounds. In terms of *Ganoderma lucidum*, there are many potential editing targets for this technology. The most direct ones are those genes closely related to triterpene biosynthesis, such as *cyp5150l8* and *cyp505d13* (Wang et al., 2019). However, to truly enhance editing efficiency, an old problem still needs to be solved: the non-homologous terminal junction (NHEJ) pathway is dominant in *Ganoderma lucidum* and often interferes with the precise modification of genes. In this regard, some studies have inhibited NHEJ by knocking out the *ku70* gene, thereby significantly enhancing the efficiency of homologous recombination (HR), and the results have indeed improved the site-specific integration of exogenous genes (Tu et al., 2021). It is worth noting that, in addition to these structural genes, some genes related to secondary metabolism regulation - such as those encoding transporters and

regulatory factors - can also serve as potential targets. Through their optimization, the synthesis efficiency of the active ingredients of *Ganoderma lucidum* is also expected to be further improved (Chen et al., 2012). CRISPR/Cas9 not only can move the "main components", but also may promote the improvement of overall performance by adjusting the "control system".

6.3 Strategies for optimizing gene knockouts or knock-ins

To make gene knockouts or knock-ins more efficient in *Ganoderma lucidum*, researchers have explored quite a few different tricks. One of the more straightforward ones is to use a CRISPR/Cas9 system guided by dual sgRNAs, which pushes target gene deletions along more effectively. This setup has already been tested and proven in genes like *ura3* and *GL17624* (Liu et al., 2020). But the adjustments don't stop there. Some scientists have even turned their attention to Cas9 itself. By inserting an intron into its coding sequence—a tweak that might seem trivial at first—they discovered it could actually boost Cas9 activity, which in turn raised the editing efficiency of genes such as *ura3*. There's also another angle: pre-assembling the Cas9 protein with its sgRNA into a ribonucleoprotein (RNP) complex outside the cell, and then delivering it directly. Experiments show that this approach can strike a nice balance, offering both high efficiency and strong accuracy (Figure 3) (Tan et al., 2023). And here's the interesting part: when these strategies are combined with ways to inhibit the NHEJ repair pathway, their benefits don't just add up—they amplify. The result is a notable overall improvement in both the precision and efficiency of CRISPR/Cas9 editing in *Ganoderma lucidum* (Tu et al., 2021; Tan et al., 2023). Put another way, each of these "small tweaks" might not look dramatic on its own, but layer them together, and suddenly you've got a real leap forward.

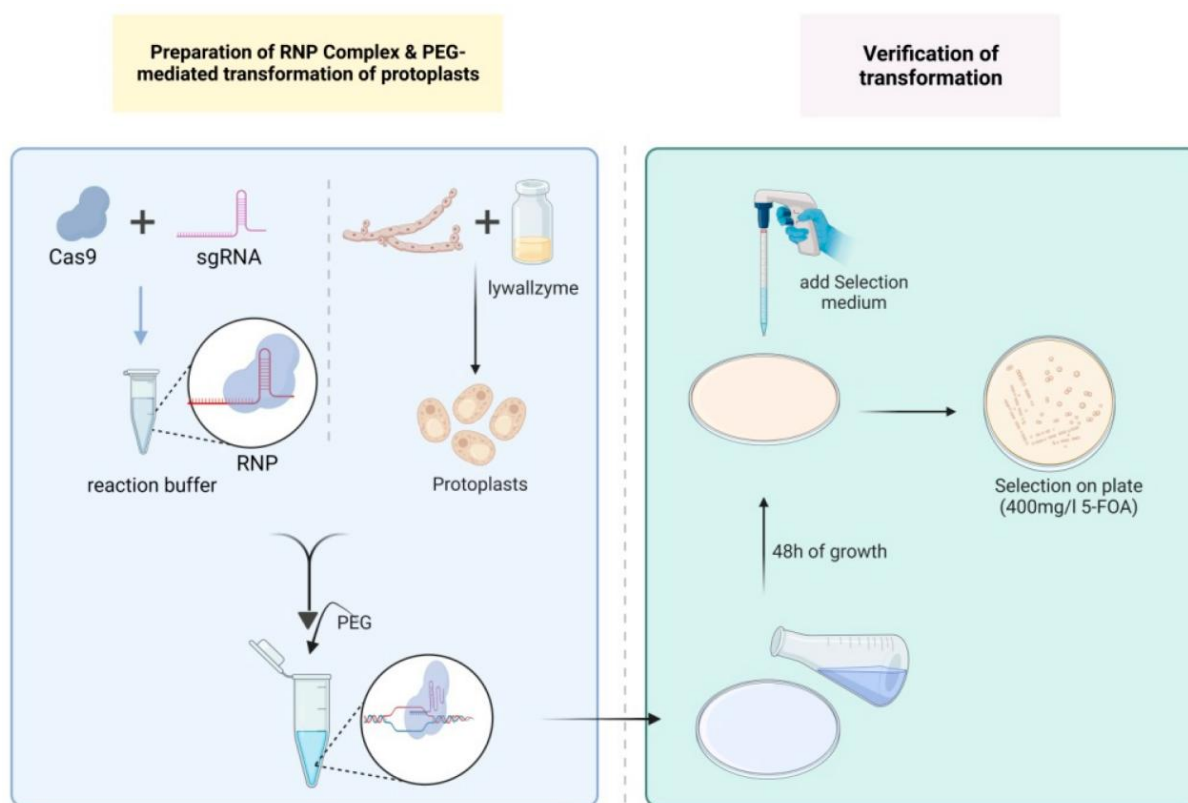


Figure 3 Schematic diagram of RNP-assisted genome editing in *G. lucidum*. (Created By Biorender: Science Suite Inc., Toronto, ON, Canada) (Adopted from Tan et al., 2023)

Image caption: The Cas9 protein and single-guide RNA (sgRNA) were assembled in reaction buffer to form RNP. The *G. lucidum* mycelia were treated with lywallzyme and protoplasts were generated. RNP complexes were added to 100 μ L protoplast suspensions (containing 107 protoplasts). After addition of Polyethylene Glycol 4000 (PEG 4000) solution, the surfactant Triton X-100 was included at a final concentration of 0.006% (w/v). The mixture was added in MM regeneration medium and incubated for 48 h. Then the medium was covered with selective MM medium containing 400 mg/L 5-FOA and incubated for 10 days (Adopted from Tan et al., 2023)

7 Metabolic Pathway Engineering via CRISPR/Cas9

7.1 Engineering metabolic pathways to increase production of target compounds

CRISPR/Cas9 technology, with its precision and efficiency, has undeniably given metabolic engineering a big push forward. And when we shift the focus to *Ganoderma lucidum*, the picture looks equally exciting-especially in terms of boosting the synthesis of valuable compounds like ganoderic acids (GAs). Take the *cyp5150l8* gene as an example. This cytochrome P450 monooxygenase sits at the heart of GA biosynthesis. When researchers knocked it out, the production of several major GA types dropped sharply. That result alone was a strong confirmation that CRISPR/Cas9 can be a reliable tool for targeted regulation of metabolic pathways (Wang et al., 2019). But the story doesn't end there. Scientists have also been asking: how can we make the process run even more smoothly? One promising answer lies in optimizing ribonucleoprotein (RNP) delivery. In *Ganoderma lucidum*, this approach has already pushed editing efficiency to a new level. What's more, it hasn't just stopped at efficiency-it's also enabled precise insertions and replacements of target sequences, offering critical technical support for rebuilding metabolic pathways (Tan et al., 2023). In short, whether it's knocking out a troublesome gene or rewriting a sequence altogether, CRISPR/Cas9 continues to broaden the horizons of *Ganoderma lucidum* research, creating opportunities that only a few years ago would have seemed out of reach.

7.2 Case examples of pathway modifications in other fungal species

The CRISPR/Cas9 technology has demonstrated its power in many fungi, especially in enhancing the synthesis of secondary metabolites, with remarkable results. Taking *Fusarium fujikuroi* as an example, researchers used this system to modify the biosynthetic pathway of gibberellic acid (GA), and finally obtained an engineered strain that could efficiently synthesize specific GA components (Shi et al., 2019). In the research on *Saccharomyces cerevisiae*, the approach goes even further. A team has attempted a multi-CRISPR /Cas9 editing strategy that can simultaneously manipulate multiple genomic loci. As a result, key intermediates in the isoprenoid synthetic pathway, such as mevalonate, were significantly accumulated (Jakocinas et al., 2015). These cases demonstrate that CRISPR/Cas9 not only can "prescribe the right medicine" in different fungi, but also shows considerable flexibility and efficiency in optimizing metabolic pathways. Whether it is for a single product or a complex pathway, it can demonstrate unique advantages.

7.3 Role of CRISPR/Cas9 in optimizing metabolic flux for bioactive compound biosynthesis

The power of CRISPR/Cas9 technology lies in its ability to directly influence the synthetic pathways of bioactive compounds through precise gene knockout, insertion or replacement. Take *Ganoderma lucidum* as an example. Some studies attempted to knock out the *ku70* gene (this gene is involved in the NHEJ repair pathway), and found that the efficiency of homologous reassembly-mediated targeted editing was significantly improved, thereby making genomic manipulation more precise (Tu et al., 2021). This modification approach not only enhances the success rate of editing but also creates the possibility of refined regulation of the metabolic network, thereby promoting the accumulation of target compounds. In addition, scientists have also knocked out genes related to secondary metabolism through CRISPR/Cas9. For example, in *Ganoderma lucidum*, after *cyp505d13* was targeted knockout, the practical value of this technology in strain improvement and metabolic optimization was further verified (Wang et al., 2019). These studies all point to one conclusion: CRISPR/Cas9 can not only help us "manually" modify the metabolic pathways of fungi, but also effectively increase the yield of high-value active products, playing an increasingly important role in fungal molecular breeding.

8 Challenges and Limitations of CRISPR/Cas9 in *Ganoderma lucidum*

8.1 Technical challenges in applying CRISPR/Cas9 to *Ganoderma lucidum*

In the process of attempting to apply CRISPR/Cas9 technology to the genetic improvement of *Ganoderma lucidum*, some technical bottlenecks that cannot be ignored have also been discovered. One of the most prominent issues is that the efficiency of homologous recombination (HR) in *Ganoderma lucidum* has always been relatively low, which directly affects the realization of precise editing such as targeted gene insertion and replacement. The main reason for this situation lies in that when Cas9 causes double-strand breaks in DNA, cells tend to prefer non-homologous end joining (NHEJ), a relatively easy but also more error-prone repair mechanism (Tu et al., 2021). What is more troublesome is that the genomic structure of *Ganoderma lucidum* itself is rather complex,

and as a basidiomycetes, its tool reserves for genetic manipulation are very limited. This undoubtedly further increases the editing difficulty (Wang et al., 2019). Of course, researchers have not failed to make breakthroughs. A team once introduced introns into the Cas9 gene to enhance its expression level, and indeed achieved certain results. But then again, this strategy still has many limitations in practical application and is far from being a "universal solution" (Liu et al., 2020).

8.2 Issues related to off-target effects and delivery methods

In genome editing, the CRISPR/Cas9 system still wrestles with a familiar problem: off-target effects. In plain terms, mutations sometimes show up where they shouldn't—outside the intended target sites. These "accidental hits," especially when they occur often, can cast doubt on the reliability of experimental results and, in practical applications, even raise safety concerns (Zhang et al., 2015). For *Ganoderma lucidum*, the hurdles don't stop there. Another tricky issue is how to actually deliver the system. Plasmid vectors are still the standard tool, but the efficiency they offer often falls short of expectations. To get around this, some researchers have tried a different route: pre-assembling the Cas9 protein and sgRNA into a ribonucleoprotein (RNP) complex outside the cell, then delivering it directly. The payoff is clear—it tends to perform better in terms of accuracy. That said, in the case of *Ganoderma lucidum*, this approach is still just getting started. It looks promising, certainly, but it will need more fine-tuning—and probably more time—before it can be considered mature enough for widespread use (Tan et al., 2023).

8.3 Regulatory and ethical considerations for genetically modified organisms (GMOs)

The use of CRISPR/Cas9 in *Ganoderma lucidum* breeding is, unsurprisingly, not free from controversy. On the one hand, it promises to push scientific progress forward and speed up industrial applications; on the other, it inevitably raises regulatory and ethical questions. After all, many countries still maintain strict rules around genetically modified organisms (GMOs). That means gene-edited *Ganoderma lucidum* strains could easily face policy hurdles in research, development, or commercialization. The concerns don't stop at regulation. Both the public and the scientific community worry about what might happen if gene-edited fungi were introduced into natural ecosystems. Could they create ecological imbalances? Or lead to long-term effects that are hard—if not impossible—to predict? These are not trivial questions. So, when advancing the use of this technology in *Ganoderma lucidum* or other edible fungi, it isn't enough to focus only on short-term gains. What's needed is balance: carefully weighing potential benefits against possible risks, and ensuring that policy decisions rest on solid scientific evidence (Chen et al., 2019; Zhang et al., 2023). In short, while the promise of CRISPR/Cas9 is hard to ignore, so too is the need for caution and level-headed judgment.

9 Future Directions in CRISPR/Cas9-Based Breeding

9.1 Prospects for using CRISPR/Cas9 in *Ganoderma lucidum* improvement

The prospects of CRISPR/Cas9 in the genetic improvement of *Ganoderma lucidum* are becoming harder to ignore—its advantages show up most clearly when the goal is to boost the synthesis of active ingredients. With this tool in hand, researchers can manipulate the genome with a precision that was previously out of reach. Knockouts, insertions, replacements—all of these techniques can be harnessed to develop new germplasms with improved traits. Take the *ku70* gene as an example. By knocking it out to block the NHEJ repair pathway, one team was able to significantly raise the efficiency of gene-directed editing in *Ganoderma lucidum*. This strategy doesn't just help in analyzing gene function—it also carries practical weight for breeding applications (Tu et al., 2021). Meanwhile, other researchers have zeroed in on ganoderic acid synthesis. By editing key genes such as *cyp515018* and *cyp505d13*, they confirmed yet again that CRISPR/Cas9 can push the yield of high-value secondary metabolites to new levels (Wang et al., 2019). Taken together, these advances sketch out a clear trajectory: CRISPR/Cas9 is well on its way to becoming a cornerstone of molecular breeding in *Ganoderma lucidum*. It not only promises strains with greater medicinal value but also paves the way for engineered varieties capable of producing more abundant active ingredients. Put differently, from early trial runs in the lab to potential large-scale applications in industry, this technology is gradually expanding the horizon of what's possible.

9.2 Integration with other biotechnologies (e.g., omics approaches)

If CRISPR/Cas9 is combined with omics technologies, research on the variety improvement and active ingredient synthesis of *Ganoderma lucidum* could move further and, more importantly, become more efficient. Omics approaches-covering genomic, transcriptomic, proteomic, and metabolomic analyses-can systematically uncover the molecular basis of active compound synthesis, giving researchers a fuller picture of the genes involved and the regulatory networks that control them. Building on this foundation, CRISPR/Cas9 can then be used for precise editing, allowing key metabolic pathways to be fine-tuned in a targeted way. The result is not only higher yields but also better quality of the desired compounds. For example, transcriptome data can be used to pinpoint genes strongly linked to ganoderic acid synthesis, which in turn provides the basis for designing more effective CRISPR targeting strategies (Wang et al., 2019; Zhang et al., 2023).

At the same time, metabolomics analysis plays an equally crucial role. It can monitor in real time how metabolites shift as a result of gene editing-making the effects of modifications clear at a glance and offering a reliable basis for further pathway optimization and improved production efficiency. Put differently, combining omics with gene editing is like giving research both a “navigation system” and an “accelerator,” charting out a clearer and more controllable path toward enhancing the functions of *Ganoderma lucidum*.

9.3 Potential for commercialization and large-scale production

The application of CRISPR/Cas9 in *Ganoderma lucidum* breeding is opening up fresh possibilities for both commercial development and large-scale production. With this tool, researchers can create high-yield engineered strains that not only provide a richer supply of raw materials for medicines and health products, but also fit neatly with the industry’s growing appetite for high value-added products. Take the optimized CRISPR/Cas9 systems, for instance. The ribonucleoprotein (RNP) delivery method has already shown high editing efficiency in experiments, laying down a solid foundation for future industrial-scale gene editing (Tan et al., 2023). What’s more, similar strategies have proven successful in other edible fungi, which makes it all the more likely that the same approach could be transplanted into *Ganoderma* breeding and propagation as well (Zhang et al., 2023). That being said, gene editing alone won’t solve everything. If CRISPR/Cas9 can be paired with modern bioprocessing technologies, the efficiency and cost-effectiveness of large-scale *Ganoderma* cultivation could be improved even further. In other words, this isn’t just about keeping up with the rising demand for *Ganoderma*’s active ingredients-it may also be the key step that carries the fungus from the lab bench to a much broader industrial stage.

10 Conclusion

The potential of CRISPR/Cas9 technology in the genetic improvement of *Ganoderma lucidum* is becoming increasingly clear, especially in terms of increasing the yield of active ingredients, showing a rather broad prospect. Its advantage lies in the ability to precisely edit genes related to high-value secondary metabolites, such as those key sites that determine the synthesis of ganoderic acid (GAs), thereby directly regulating metabolic pathways. A typical example is the knockout experiment on the *cyp5150l8* gene. The results showed that the contents of the four major gases decreased significantly accordingly, which also proved from the side the effectiveness of CRISPR/Cas9 in targeted regulation of metabolic pathways. Moreover, not only that, this system has also been applied to the editing of other functional genes such as *cyp505d13*, further demonstrating its flexibility and practical value in the improvement of *Ganoderma lucidum* strains and biotechnology research and development. Therefore, CRISPR/Cas9 is not merely a “scissors” tool, but rather offers more controllable and efficient possibilities for molecular breeding and functional development of *Ganoderma lucidum*.

In addition, researchers have experimented with inserting introns upstream of the Cas9 gene to boost its expression. Interestingly, what seemed like a small tweak ended up making a big difference: the editing efficiency of CRISPR/Cas9 in *Ganoderma lucidum* improved markedly, and the construction of gene deletion mutants became noticeably smoother. This kind of progress provides solid technical support for both functional genomics research and for dissecting the biosynthesis of active ingredients. It’s also worth noting that the use of CRISPR/Cas9 goes far beyond *Ganoderma lucidum*. The technology has already been successfully applied in a

range of edible fungi, showing that it carries strong potential in fungal molecular breeding more broadly. Put differently, using CRISPR/Cas9 to increase the yield of bioactive compounds is no longer just a theoretical idea—it's a path that researchers are actively walking.

Gene editing technology, especially the CRISPR/Cas9 system, is showing increasingly broad prospects in the research of medicinal fungi. Its advantage lies in its ability to achieve high-precision insertion, deletion and replacement of genes, providing a brand-new path for enhancing the synthesis capacity of active ingredients. Take *Ganoderma lucidum* as an example. If the NHEJ repair pathway is inhibited, the efficiency of gene-directed editing can be significantly improved, and more precise genomic manipulation can also be made possible. This breakthrough is not only an improvement at the technical level, but also means that we can go further in the optimization of metabolic pathways, thereby cultivating engineered strains that can efficiently synthesize specific active ingredients. It has opened up new imagination space for the development and application of medicinal fungi.

In addition, the CRISPR/Cas9 system—thanks to its relative simplicity and high efficiency—has gradually established itself as a powerful way to get around the limits of traditional genetic manipulation. Compared with older methods, which are often slow and not particularly precise, its strengths are hard to miss. Even so, its value doesn't just lie in being “faster and more accurate.” As research continues to dig deeper, CRISPR/Cas9 is likely to keep driving new ideas in fungal biotechnology, opening doors to active compounds that not only are novel but also carry real therapeutic promise. Put another way, this technology isn't just patching a technical shortfall—it's also creating room for applications that, until recently, would have been difficult to imagine.

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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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