# Tools and Approaches for the Genetic Engineering of Basidiomycete Fungi

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## Abstract

Basidiomycete fungi are organisms of biotechnological interest because of their capabilities to produce natural products, their ability to degrade lignocellulose in e. g. wood, their nutritional value, and their potential use in building materials. The scope of available gene editing tools to study enzymatic pathways and improve production in these organisms is less advanced than for bacteria and ascomycete fungi. New developments with CRISPR-Cas have expanded possibilities for gene editing. This review discusses the development and state of the art of genetic engineering methods in basidiomycetes. The most important developments relate to development of transformation systems, selection markers (or marker-free systems), and CRISPR-Cas mediated homology directed repair (HDR). These tools are most advanced for *P. ostreatus* (oyster mushroom) and *G. lucidum* (reishi), but can be expanded to the understudied but highly relevant *L. edodes* (shiitake mushroom).

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**Statement on the use of AI tools/Large Language Models (LLMs)**

No part of this thesis was written, improved or rephrased by the use of AI tools and/or Large Language Models. No ideas presented in this thesis were generated by AI and/or Large Language Models. To aid the search for research papers on the discussed topics, the EvidenceHunt AI tool was used (www.evidencehunt.com).

# Introduction

Basidiomycetes are one of the most important and diverse groups of fungi, and the phylum contains approximately 30% of all described fungal species [Schmidt-Dannert, 2016]. They are characterised by their ‘clubs’, the basidium that produces its haploid spores. Basidiomycetes have an unique life cycle, and their mycelium is able to proliferate in both monokaryotic and dikaryotic states. The division of basidiomycetes contains many common edible mushrooms, along with common plant pathogens. Their ability to degrade organic matter makes them unique carbon recyclers that influence the environment on a global scale. Besides this, basidiomycetes provide a large amount of enzymes interesting for biocatalysis, and produce countless useful natural products [Schmidt-Dannert, 2016][Alberti et al., 2020].

The use of fungi in biotechnology expands, and they are increasingly harnessed to produce new and existing drugs [Alberti et al., 2020]. Advancements in genetic engineering lead to the discovery of new products, and the development of fungal strains that produce higher yields of the desired compounds. Although production of compounds by engineered ascomycetes is already a multi-billion dollar industry, advancements in basidiomycetes lag behind [Cairns et al., 2018][Nielsen, 2013]. A quick search on PubMed teaches us that there are approximately six times more papers mentioning ascomycetes than basidiomycetes. Secondly, engineering in basidiomycetes is more difficult because of their slow growth rates and complex genomes [Alberti et al., 2020]. The combination of the large biotechnological potential, but limited amount of research and tools available indicates the necessity of the development of new tools.

Recent developments in CRISPR-Cas engineering have revolutionised genetic studies. While CRISPR-Cas is originally an adaptive immunity mechanism of bacteria against bacteriophages, it can nowadays be used for specific gene editing. The most widely used CRISPR system is CRISPR-Cas9, where the Cas9 protein can create a double-strand break (DSB) in a genomic sequence matching a guide RNA (gRNA) after a PAM-sequence (NGG). Consequently, through cellular DNA repair mechanisms new DNA can be inserted at the location of the DSB if the insert contains ends homologous to the region around the double-strand break (homology directed repair, HDR). In this way, gene knockouts or edits can be achieved [Swartjes et al., 2020]. CRISPR is currently being widely applied to all sorts of organisms, and it is also transforming the methods of genetic engineering in basidiomycetes.

This review will discuss the current state of non-CRISPR-based and CRISPR-Cas-based genetic engineering in basidiomycete fungi. It will aim to outline the most pivotal research topics and developments. Because a major challenge in the field of basidiomycete engineering is transferring existing knowledge to new organisms [Liao et al., 2021], this paper will have a special focus on the shiitake mushroom, *Lentinula edodes*. *L. edodes* is highly promising for bioengineering, because of its

wide scope of properties including wood-degradation, anti-cancer and immunomodulatory properties, and nutritional value [Chen et al., 2016][Gariboldi et al., 2023][Roszczyk et al., 2022][Łysakowska et al., 2023]. The research papers on the engineering of this mushroom however can be counted on the fingers of one hand. We will therefore have a look into the existing literature on this mushroom, so that in the light of the state of the art technologies in other mushrooms some future perspectives can be drawn for its further development.

In the first section of this review, general methods, topics, and challenges in the engineering of basidiomycetes will be discussed. The second section will focus on the two basidiomycete organisms with the most biotechnological relevance, and the most advanced engineering toolbox available to date, *Pleurotus ostreatus* and *Ganoderma lucidum*. The far more limited amount of research available on *L. edodes* will be discussed in the third section. Finally, the considered methodologies will be weighed in the context of remaining challenges and future prospects and applications of fungal engineering.

**Figure 1** From left to right: *P. ostreatus*, *G. lucidum*, *L. edodes* growing on wood. Respective photo credits Dominicus Johannes Bergsma, Nina Filippova and 'Frankenstoen'. Wikimedia commons Creative Commons Attribution 4.0

## Overview of the genetic engineering toolbox and developments

Genetic engineering aims to alter the DNA of organisms in order to change their genetic composition for the production of natural products (or other purposes). It can be done by disrupting genes, mutating them, or transforming new genetic material into the cell. Transformation can be done transiently, with a separate plasmid not being integrated into the genome, or transgenically, where the host genome is modified. This

section will discuss the artefacts of the main methodologies used in the genetic engineering of basidiomycete fungi.

The fungal cell wall is a major obstacle for transforming DNA into cells, which is why most transformation methods rely on the formation of protoplasts. Protoplasts are mostly formed by enzymatic lysis of the cell wall, which can be performed by enzymes from for example *Trichoderma harzianum* [Lim et al., 2021]. Obtaining sufficient protoplasts is often challenging, and controlling the osmotic stability of the environment is important to prevent cell rupture [Li et al., 2017].

Transformation of protoplasts (see figure 2) in the presence of polyethylene glycol (PEG) is the most common method for fungal transformation. For each fungal species, a specific protocol has to be developed to determine the optimal plasmid concentration, PEG concentration, and incubation times [Lim et al., 2021]. Besides chemical methods, a physical method like electroporation is commonly used. In electroporation, the permeability of the cells is increased by applying electrical waves. This method is relatively simple, but the risk of damaging the cell membrane and thereby killing the cells is high [He et al., 2017][Li et al., 2017]. Other methods, like liposome-mediated transformation, viral-vector mediated transformation, biolistic transformation, and shock-wave-mediated transformation, will not be discussed in this review but are described in He et al. (2017) and Li et al. (2017). Another method, Agrobacterium-mediated transformation, will be discussed in section 3. In all methods, a major challenge is the regeneration of viable cells after transformation. For many ascomycetes, elaborate protocols for transformation are already available [Li et al., 2017]. However, suitable protocols still have to be developed for most basidiomycetes.

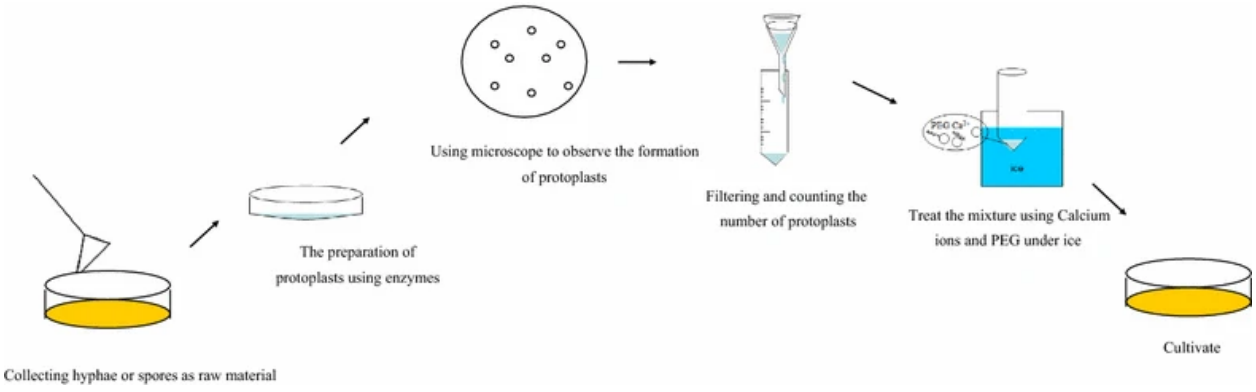


**Figure 2** The formation of protoplasts for fungal genetic engineering, figure is adapted from Li et al., 2017.

In order to achieve successful transformation, suitable selection markers are necessary. The two most applied selection markers in fungi are hygromycin B and carboxin resistance. Resistance against hygromycin B is obtained by adding the gene for hygromycin phosphotransferase (hph) to the cells, and this system is already being used for four decades [Kilaru et al., 2009][Blochlinger and Diggelmann,

1984]. However, the hph gene is poorly expressed in some fungi, possibly due to its bacterial origin [Xu and Zhong, 2015]. Carboxin resistance is obtained by expressing a resistant mutant of the target protein of carboxin, a succinate dehydrogenase. This marker system is more easily expressed in some fungi because it is a homologous system and has been used in several organisms, including *G. lucidum* and *Coprinopsis cinerea* [Xu and Zhong, 2015]. The amount of available selection markers is however very limited and the development of new markers is desperately needed (some progress is described in later sections of this paper, an overview can be found in table 1).

**Table 1** Selection marker systems used for basidiomycete fungi. The list of organisms for which they are used is limited to the organisms described in this paper, the markers are also used in other basidiomycetes

| Fungicidal compound | Resistance gene | Use |
|---|---|---|
| Hygromycin B | Hygromycin phosphotransferase | *P. ostreatus* not suitable for all organisms because of poor expression |
| Carboxin | Succinate dehydrogenase mutant | *P. ostreatus*, *G. lucidum*, and *L. edodes, C. cinerea* |
| Nourseothricin | Nourseothricin N-acetyl transferase | *P. ostreatus* |
| 5-fluoroorotic acid | pyrG disruption | *P. ostreatus*, *L. edodes*, marker recycling |
| 5-fluorocytosine | FCY | *P. ostreatus*, *C. cinerea* |

Genome editing and gene disruption are generally performed by creating a DSB in the DNA and consequently hijacking the cell's own DNA repair mechanisms. The two main repair mechanisms are homologous recombination (HR) and nonhomologous end joining (NHEJ). HR is a repair pathway that uses a homology template to repair the DNA. Providing an alternative template with a mutation or gene of interest can be used to modify or insert genes. NHEJ ligates the DSB without a template, often causing random mutations in the process. It can therefore be used to knock-out genes, but it often has off-target effects. Therefore, HR enables for more accurate gene editing. HR is however notoriously difficult in basidiomycetes. Cells naturally prefer the NHEJ method and therefore the HR efficiency (the percentage

of DSBs repaired by HR) in most basidiomycetes is close to 0% [Salame et al., 2012]. Some strain engineering developments to improve HR efficiency are described in the next section.

The greatest achievement of CRISPR-Cas9 is that through homology directed repair it enables gene editing that would otherwise have been impossible in basidiomycetes. CRISPR-Cas can create double- or single stranded DNA breaks on very specific places in the genome determined by the sequence of a single guide RNA (sgRNA). This sgRNA can be expressed on the same plasmid as CRISPR or it can be added separately through transformation [Ding et al., 2020][Liao et al., 2021]. This sgRNA template allows for the creation of very specific gene disruptions, which is one of the main methods to study the role of enzymes *in vivo*. Through CRISPR-mediated HDR, several studies have now successfully inserted DNA fragments into fungal genomes [Alberti et al., 2020][Vonk et al., 2019]. While CRISPR technologies in ascomycetes and yeasts are already quite common, CRISPR research in basidiomycetes is limited and only thoroughly developed in a couple of model organisms [Liao et al., 2021]. The occurrence of a-specific NHEJ remains a challenge to be tackled in future research [Alberti et al., 2020].

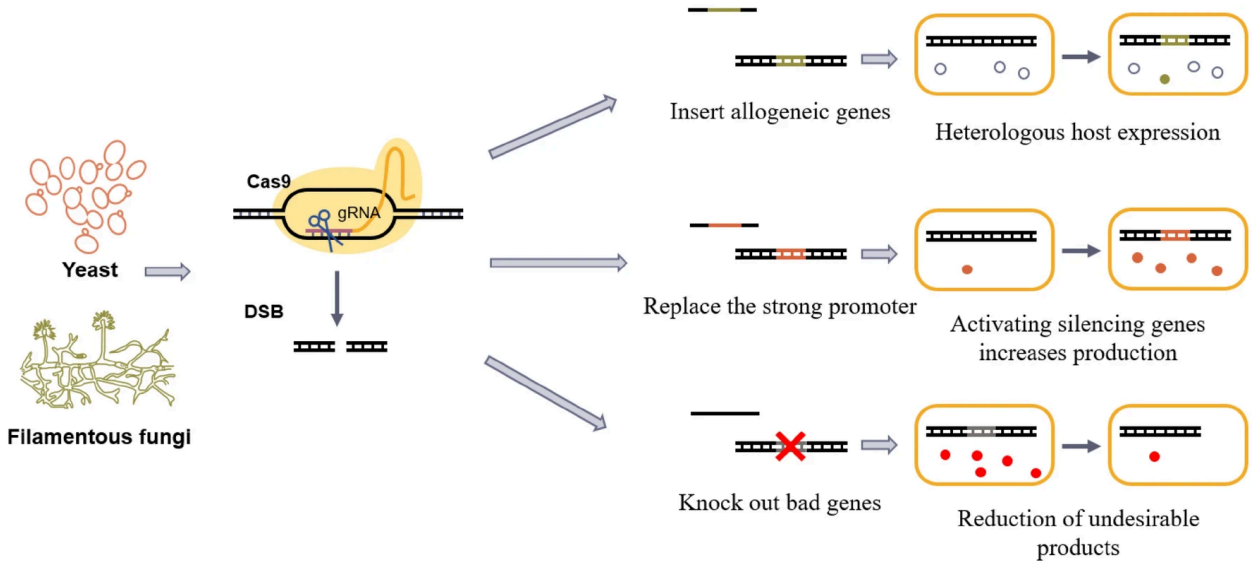


**Figure 3** Applications of CRISPR-Cas9 in metabolic engineering, the figure is adapted from Liao et al., 2021.

Studies into enzyme functions, pathways and transformation systems are usually performed by a combination of the methodologies mentioned above. Besides this, additional artefacts like choosing the right promoters for expression of enzymes and/or CRISPR RNA (crRNA) will play a role in genetic engineering experiments. In some cases, heterologous expression of pathways from basidiomycetes in better studied ascomycetes and yeasts might still provide a suitable alternative to developing genetic tools in basidiomycetes [Alberti et al., 2020].

## *Pleurotus* and *Ganoderma*, the frontrunner organisms

The two main basidiomycete organisms of scientific interest are *P. ostreatus* and *G. lucidum*. Among the division of basidiomycota, these two organisms are studied most extensively and are farthest progressed in the development of genetic tools. We will now have a look into the molecular tools and topics described in the previous paragraphs and their state of development in those two organisms.

*P. ostreatus* (oyster mushroom) is one of the most common edible fungi. First genetic studies into this organism were done with the development of resistance markers, such as carboxin and nourseothricin [Nakazawa et al., 2024]. One of the most important developments in the genetic engineering of *P. ostreatus* was the development of a ku80 knockout strain. This system, developed by Salame et al. targets the ku80 gene which is crucial in forming a protein complex with an exonuclease and DNA ligase needed for NHEJ. After successful disruption of the ku80 gene, this Δku80 decreased the rate of NHEJ from 97.8% to 0%, leading to a 100% HR efficiency after addition of foreign DNA [Salame et al.. 2012]. This allowed already before the development of CRISPR-Cas methods for a variety of gene editing possibilities.

The usefulness of this ku80 deletant strain was shown by a recent study into chitin synthases by Schiphof et al., 2024. Chitin synthases are membrane proteins responsible for synthesis and modelling of chitin, one of the major components of the fungal cell wall. After identifying the genes of chitin synthases in *P. ostreatus* by molecular genetic analysis, Schiphof et al. tried to disable several of these genes, namely chsb2, chsb3 and chsb4. Through homologous recombination, a disruption cassette was inserted into the respective chsb genes of a ku80-disrupted strain. The authors observed that the chsb mutants did not have a different growth rate compared to the wild type, but the mycelium was more thinly distributed and the morphology of the hyphae was altered. This study therefore showed that disabling NHEJ is an important step in fungal engineering. Understanding enzymes involved in the synthesis of fungal mycelia and cell walls is very useful for the use of fungal mycelium in building materials [Elsacker et al., 2020], as will be discussed in section 4.

The development of CRISPR-Cas methods provides a convenient alternative to the use of ku80-disrupted strains for gene editing and is recently being implemented in *P. ostreatus*. Since *P. ostreatus* is an edible mushroom and cultivated for both nutritious purposes and building materials it is extremely useful to have genetic engineering methods for not just lab strains such as the ku80 deletant. In addition to plasmid based CRISPR-Cas methods, CRISPR engineering can be done by pre-assembling the CRISPR-Cas ribonucleoprotein (RNP) complex before adding it to the cell. The major problem with the RNP-based method is the lack of a selection mechanism, making it only useful for genes that could be selected for. The first attempt to solve this problem was done in 2023 by Boontawon et al. The authors of this study

assembled a system that is through a RNP complex capable of introducing mutations in two different genes. In this study both these mutations could be selected for, but in the future one of these genes can then be a selection gene, while the other can be a target gene for a study. The authors isolated 76 strains containing one mutation, but only three of those also had the second mutation. So despite being successful in obtaining double mutants, the efficiency of obtaining this double mutant was very low (3.9%). This makes the system not yet available for use without genomic sequencing. However, it brings us one step closer to creating a marker system that would enable using RNPs on a larger scale in molecular genetics.

Despite advances in RNP-based methods, transformation of CRISPR-Cas genes is still the main method for CRISPR-based engineering in *P. ostreatus*. The most important benefit of CRISPR-Cas is the possibility of introducing multiple mutations. This is limited with 'traditional' genetic engineering because of the limited amount of selection markers, but highly useful since many pathways of biotechnological interest are influenced by multiple genes [Xu et al., 2022]. In 2022, two studies were performed showing the current scope of solutions for obtaining multiple mutations. The first was performed by Xu et al., who used polycistronic sgRNAs (i. e. containing multiple genes on one sgRNA). By creating a polycistronic array of sgRNAs with endogenous tRNA editing the authors of this study managed to obtain three simultaneous mutations in genes involved in lignin degradation.

The need for marker systems will always limit the possibilities for multiple-gene editing, which is why a second study investigated the development of a marker-free system. In a marker-free system, the amount of gene edits is not limited by the amount of available markers, and no extra DNA edits are necessary to provide a marker system. A 2022 study by Koshi et al. exploited the difference between transient and transgenic transformation to create a marker-free system. They expressed a plasmid containing CRISPR-Cas genes, sgRNA and a hygromycin resistance gene (hph). The hygromycin resistance marker was used to select for clones that had taken up the plasmid. In these clones, gene editing occurred through CRISPR-Cas mediated HDR. Because the plasmid was transiently and not transgenically expressed, the researchers obtained clones that lost their hygromycin resistance. These clones thus did not contain any traces of the selection marker, but did contain the introduced gene edits. This system is of course not completely free of the use of a selection marker, but the same marker can be used multiple times to enable multiplex gene editing.

*G. lucidum* is the second most studied basidiomycete organism, being of biological interest because of a class of compounds called the ganoderic acids, which are known for their many medicinal purposes [Azi et al., 2024]. Genetic engineering in *G. lucidum* has been developed along similar lines as in *P. ostreatus*, and its genetic engineering pathways are shown in figure 4. However, gene editing is more challenging since CRISPR-Cas9-mediated gene insertion is difficult and inefficient [Tu et al., 2021]. CRISPR-Cas is

here however suitable for gene disruptions, which is why Tu et al. performed a gene disruption of a ku70 gene (similar to Salame et al., 2012, in *P. ostreatus*) with CRISPR-Cas to increase HR efficiency by decreasing NHEJ activity. The researchers observed an almost thirtyfold increase in gene insertion with HR in the ku70 knockout strain compared to CRISPR-Cas mediated insertion.

Like in *P. ostreatus*, multiplexing (editing several genes at the same time) is also a challenge in *G. lucidum*. Azi et al. (2024) propose the use of another CRISPR system, CRISPR-Cas12a (also called Cpf1), for multiplexing in *G. lucidum*. CRISPR-Cas12a systems differ from Cas9 systems in their ability to produce DNA breaks with an overhang, and a more simple multiplexing mechanism, making use of crRNA arrays [Zetsche et al., 2017]. Despite Azi et al. claiming that CRISPR-Cas12a is very suitable for *G. lucidum* because of the organisms high genomic GC content, there have been no examples so far of studies using it in any basidiomycete. CRISPR-Cas12a is now mostly being used in mammalian cells, some bacteria and two ascomycete fungi [Yang et al., 2023][Huang and Cook, 2021][Abdulrachman et al., 2022].

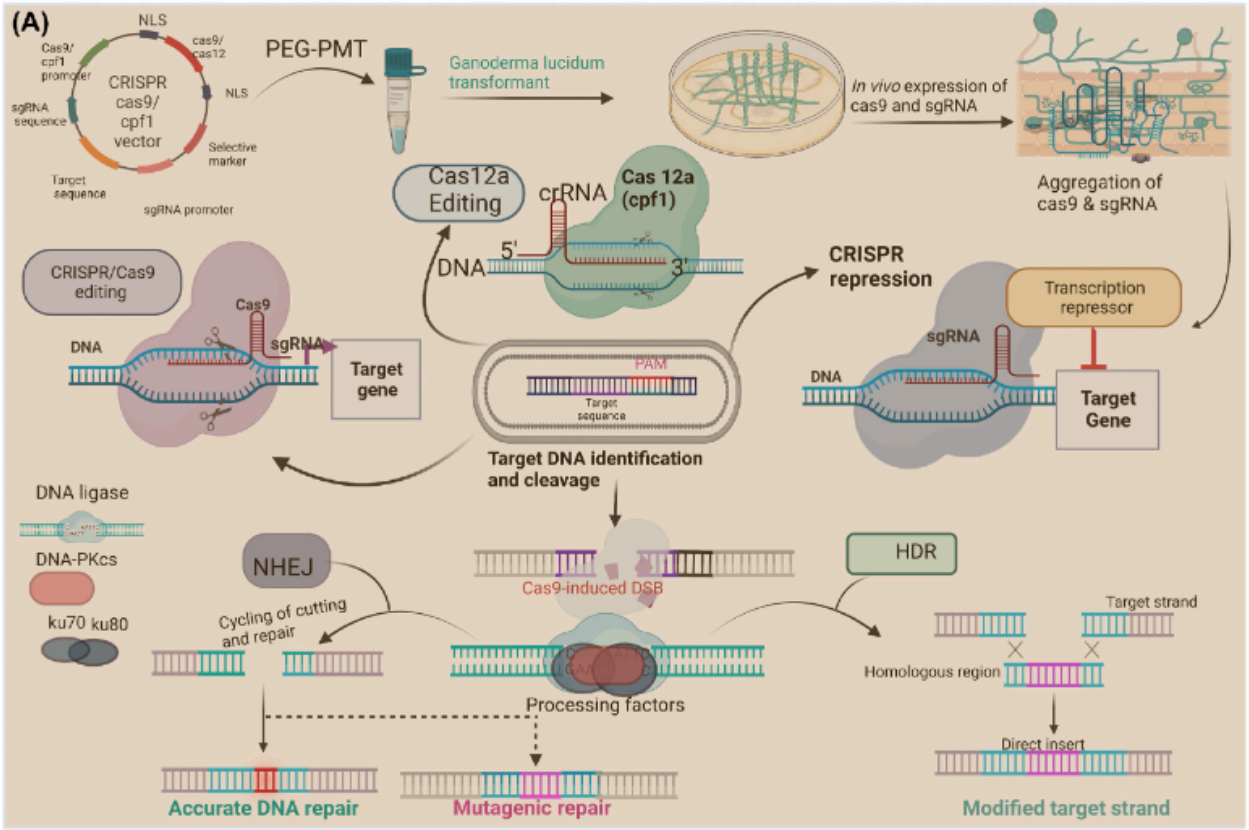


**Figure 4** Pathways and strategies of CRISPR engineering in *G. lucidum*, figure is adapted from Azi et al., 2024

The main purpose of genetic engineering in *G. lucidum* is to increase its production of ganoderic acids, the triterpenoids of medical interest produced by the mushroom. Most studies on genetic engineering in

*G. lucidum* are still in an earlier phase, focussing on developing tools rather than focussing on 'final' engineering goals. However, there are a few studies which have succeeded in slightly increasing the yield of ganoderic acid production. A study by Xu et al. from 2012 managed to overexpress a 3-hydroxy-3-methylglutaryl coenzyme A reductase by transformation. With this they managed to increase the total yield of ganoderic acids, but overproduction of specific compounds is limited by the lack of understanding of downstream enzymatic pathways [Xu et al., 2015]. A more recent example by Fei et al. (2019) shows how overexpression of a farnesyl diphosphate synthase similarly yielded increased ganoderic acid production. Studies investigating genetic engineering for ganoderic acid production are thus based on classical transformation and overexpression techniques and are mainly limited by the lack of understanding of the enzymes involved in the complicated pathways of ganoderic acid production.

The limited amount of research successful in engineering *G. lucidum* for increased ganoderic acid production has led the authors of a study in 2021 to explore the heterologous expression of a ganoderic acid in *Saccharomyces cerevisiae*. Wang et al. (2022) managed to express a P450 enzyme producing a new ganoderic acid called DHLDOA. This heterologous expression of ganoderic acids could provide an alternative to the challenges that gene editing in *G. lucidum* poses.

## Expanding the scope of applications in *Lentinula edodes*

*L. edodes* is a widely cultivated, edible mushroom that has recently received attention because of its potential to produce medicinal compounds, similarly to *P. ostreatus* and *G. lucidum* [Gariboldi et al., 2023][Łysakowska et al., 2023]. In 2016, its full genome was published, revealing that the species also contains many enzymes related to lignolytic pathways [Chen et al., 2016]. This discovery of 101 lignocellulolytic enzymes highlighted the potential of *L. edodes* for wood degradation and agricultural waste removal. As an edible mushroom with medicinal and lignocellulotic capacities, the biotechnological potential is not less than that of the better studied basidiomycete organisms described before. However, molecular genetic studies are significantly less advanced. Therefore, some recent advances on the genetic engineering of *L. edodes* will now be discussed, concerning both 'traditional' engineering and CRISPR-based. By studying present technologies, we will gain more understanding in steps that are and could be taken to improve the engineering of this organism.

A clear example of the genetic engineering of *L. edodes* was published by Sato et al. in 2019, who studied transformation vectors for altering the expression levels of the tyrosinase gene. This tyrosinase plays an important role in the browning of the fruiting body (i. o. w. decay of the edible mushroom), and knocking it out could yield a strain that is easier to conserve for consumption. By homologous overexpression of

the tyrosinase gene, the authors showed that they could increase the tyrosinase activity in both the mycelium and the fruiting bodies. To downregulate the tyrosinase, an antisense vector was used. Antisense RNA is RNA complementary to the gene RNA and is capable of downregulation translation by interacting with the coding mRNA [Xu et al., 2018]. The vectors employed in this study, using a hygromycin resistance gene and a chitin synthase promoter, show the suitability of this transformation method for gene overexpression and repression in *L. edodes*.

A second significant advancement in the transformation methods of *L. edodes* was also achieved in 2019 by Yan et al. These researchers investigated the potential of transformation mediated by *Agrobacterium tumefaciens*. Agrobacterium-mediated transformation (AtMT) is a widely used transformation method in plants and fungi relying on the capacity of *A. tumefaciens* to insert DNA in the host genome [Idnurm et al., 2017]. Yan et al. transformed a *L. edodes* strain by inserting hygromycin-B resistance genes into the genome. They showed that stable transformants could be obtained in both monokaryotic and dikaryotic strains (more on the fungal life cycle and karyotic state in the penultimate paragraph of this section). Because of this methodology, it is possible to create inserts in the *L. edodes* genome with high transformation efficiency, giving an advantage over PEG transformation, which mainly results in transient transformation.

There are two examples of successful CRISPR engineering in *L. edodes*, both aiming to expand the scope of the genetic tools available for this organism. The first one was a study with a plasmid-based CRISPR system targeting the mating type gene HD1 [Moon et al., 2021]. In tetrapolar basidiomycetes like *L. edodes*, mating type determines whether different monokaryotic hyphae are compatible with each other to form dikaryotic (n + n) mycelium (see figure 5). Moon et al. created a plasmid with an endogenous promoter, hygromycin resistance, and CRISPR-Cas9 genes with several gRNAs. HD1-mutant protoplasts showed significant downregulation of HD1 RNA and downstream genes [Moon et al., 2016]. Compared to the state of the art of CRISPR in other organisms this is a small step, but for *L. edodes* it is a significant advancement.

The second study investigating CRISPR in this organism investigated the possibility of marker recycling. Marker recycling would enable multiplexing in multiple consecutive transformation experiments. Although this is not as easy as multiplexing in one step, as has been achieved for *P. ostreatus*, it would still be great progress to the engineering of *L. edodes*. The study by Kamiya et al. (2023) focusses on the pyrG gene, which normally causes cell death when cells are exposed to 5-fluoroorotic acid (5-FOA). Knocking out of pyrG is thus a method of obtaining 5-FOA resistance. Like in other basidiomycetes, the HR rate in *L. edodes* was too low to allow gene editing without CRISPR-Cas. By co-transformation of a CRISPR-Cas9 vector and a PyrG disruption vector increased HR efficiency was obtained, resulting in 5-FOA resistant strains.. Another interesting finding by Kamiya et al. was the fact that their ΔPyrG strain

required uracil to be present in the growth medium, while this is not the case for the wild-type strain. A plasmid containing a PyrG gene can consequently be used as a selection marker for growing the ΔPyrG organism on a uracil-depleted medium. The fact that the ΔPyrG strain is 5-FOA resistant but an uracil auxotroph (i. e. unable to synthesise uracil) shows that this system has the potential to be a marker recycling system.

This system of marker recycling with a ΔPyrG strain has previously been established in *P. ostreatus* by Nakazawa et al. (2016). The authors studied the system described above and possible marker recycling in the gene editing of manganese-dependent peroxidases (MnP genes), enzymes playing an important role in lignin degradation. Development of marker recycling systems for studying these enzymes is relevant, since single-knockout of MnPs did not alter the lignin-degrading phenotype. The study managed to obtain multiple gene MnP edits while regaining prototrophy (i. e. the opposite of auxotrophy) after transformation of a PyrG gene. The acquisition of this marker recycling system in *P. ostreatus* shows the potential of multi-gene editing through this method of *L. edodes*.

A big challenge in the genetic engineering of *L. edodes* and other basidiomycetes is posed by the complicated life cycle of these organisms. Basidiomycete spores start off as monokaryotic (i. e. one nucleus per cell), haploid organisms. After mating with a complementary mating type, monokaryotic hyphae can form dikaryotic hyphae (n + n). This dikaryotic hyphae can under the right conditions form a fruiting body (mushroom). From the basidia in the fruiting body, new spores are released. The complete life cycle of basidiomycetes is depicted in figure 5. Monokaryotic cells are easiest for gene editing, and obtaining the same mutations in both nuclei of dikaryotic cells is challenging [Liao et al., 2021]. Additionally, a successful mutation in a recessive allele might be invisible in the phenotype after mating. The transformation of dikaryotic mycelium of *L. edodes* by AtMT described before is an important step in tackling this problem. Furthermore, the cultivation of fruiting bodies (the mushrooms) is challenging, but highly relevant for studying edible fungi like *G. lucidum*, *P. ostreatus* and *L. edodes*. While mycelium grows on standard lab media, fruiting bodies have to be cultivated on alternative media, such as the sawdust used by Sato et al. (2019). To go from an engineered monokaryotic fungus, to the dikaryotic and fruiting body stages, successful dikaryon formation through mating has to be obtained. For a review on mechanisms involved in dikaryon formation, see Kruzel and Hull, 2010. Further investigation into the effect of the life cycle stage on engineering of *L. edodes* would greatly benefit future genetic research.

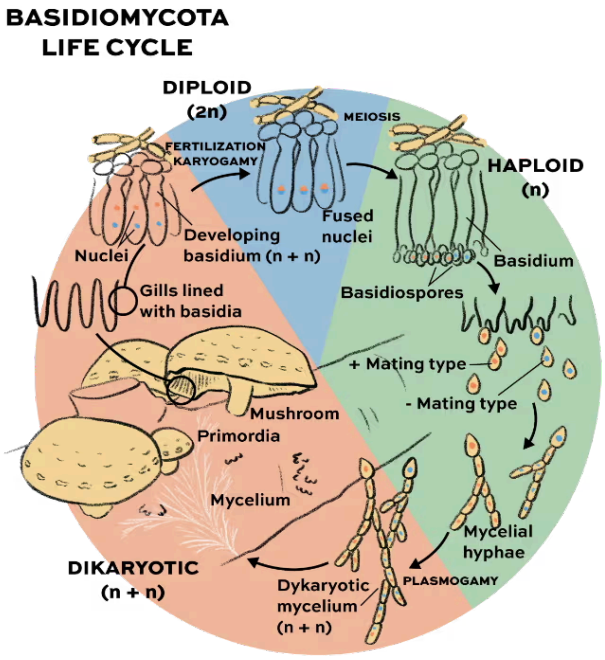


**Figure 5** The life cycle of *L. edodes*. The figure is adapted from The North Spore, a North American centre for sustainable mushroom cultivation (https://northspore.com/blogs/the-black-trumpet/introduction-to-fungal-biology).

Looking back, we can now assess the complete molecular toolbox available for *L. edodes*. Two successful transformation methods have been described, one with classical plasmid vectors and one with Agrobacterium-mediated transformation. CRISPR-Cas has been used to increase the fungus' HR efficiency. CRISPR-Cas has also been used to develop a new marker selection system based on previous research in *P. ostreatus*. However, as many phenotypic changes are affected by multiple genes, the development of a marker recycle system would further aid genetic research. Marker-free, RNP-based methods could provide an alternative to the difficult development of selection systems. Lastly, investigation in the control of the life cycle and optimal media to cultivate the different stages of the fungal life cycle are needed to expand the scope of genetic engineering in *L. edodes*.

# Possibilities and perspectives

Development of new molecular genetic techniques would not be necessary if it were not for the broad potential of applications of basidiomycetes. This final section will discuss three of the most important ways in which these organisms can be implemented to tackle future global challenges. A concise overview of biotechnological properties will be given, but it is far from complete as the scope of applications of basidiomycetes is too large to describe in a single chapter.

Natural products derived from fungal secondary metabolites have a great potential for being used in medicine. In a 2023 study, it was shown that eight edible fungi, including *P. ostreatus, G. lucidum, and L. edodes*, produced secondary metabolites active against breast cancer [Gariboldi et al., 2023]. These mushrooms exhibit general antitumour effects, along with immunomodulatory effects [Łysakowska et al., 2023]. Polysaccharides from *L. edodes*, especially β-glucans found in the cell wall, are shown to be capable of stimulating or repressing the immune system in several conditions [Roszczyck et al., 2022]. The terpenoids produced by *G. lucidum* (the ganoderic acids) exhibit, besides aforementioned properties, also activity against conditions such as HIV, high blood pressure, and high cholesterol [Xu and Zhong, 2015].

Another basidiomycete that is gaining attention for its medicinal properties is *Hericium erinaceus* (Lion's mane mushroom). This mushroom has been known for millennia in Chinese traditional medicine for its neuroregenerative properties, and studies have shown secondary metabolites with potential activity against Parkinson's disease, Alzheimer's disease, depression disorder and some types of cancer [Ghosh et al., 2021][Chong et al., 2019][Gregory et al., 2021]. The discussion of the state of genetic engineering methods in *Hericium erinaceus* was beyond the scope of this paper, but would be a very promising subject for future inquiries.

Finally, basidiomycetes not only produce drugs targeting human diseases, but also produce a wide range of compounds with antibacterial or antifungal properties. Examples of antifungal compounds from organisms described in this article include ganodermin, pleurostrin, and lentin, but the complete list with antimicrobial compounds produced by basidiomycetes is much longer [Sivanandhan et al., 2017]. The still partially undiscovered realm of fungal secondary metabolites appears to be a goldmine for future drug developments.

Basidiomycetes are not only interesting because of what they produce but also for what they can degrade, as many basidiomycetes have lignocellulose degrading properties. The capacity to degrade lignin, exhibited by white-rot fungi (a diverse class of basidiomycetes including *P. ostreatus* and *L. edodes*) gives these organisms the rare ability to degrade wood and inedible agricultural waste [Atiwesh et al., 2022]. Degradation of wood happens by enzymes such as laccases and manganese-dependent peroxidases

(MnPs)[Knop et al., 2015]. As described before, *P. ostreatus* and *L. edodes*, among other species, can express these enzymes and the ability to grow nutritional and medicinal mushrooms from (agricultural) waste is a major potential of basidiomycetes. Additionally, ligninolytic enzymes can be engineered to expand the scope of their substrates, as was described previously by Asemoloye et al. (2021).

A third, more recently developed application of basidiomycete mycelium is its use in biomaterials. The cell walls of hyphae can provide remarkable structures. Fungal mycelium can be grown and moulded in both rigid and flexible structures, having applications in packaging and construction. Current research on fungal biomaterials is focussing on the over thirty factors in the production process that can affect the material properties. Among these factors are the choice of species, cell wall composition, and growth conditions [Elsacker et al., 2020]. Despite the need for more research on both molecular and supramolecular structures of fungal hyphae, biomaterials can already be used in construction and soft materials like fabrics and packaging materials [Gandia et al., 2021][Raut et al., 2021]. Figure 6 shows an overview of the processing of fungal materials for use in construction.

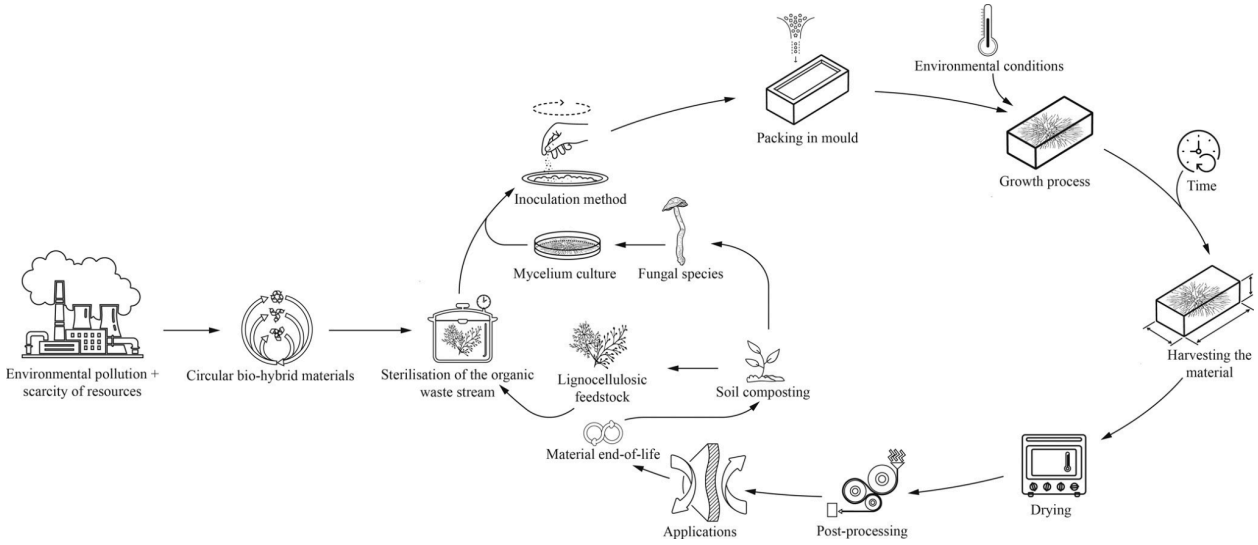


**Figure 6** The cycle of producing fungal building materials, the figure is adapted from Elsacker et al., 2020.

# Concluding remarks

This review has shown that like in many organisms, research on genetic engineering in basidiomycetes is focused on developing methods for transformation and selection. The development of CRISPR-Cas9 systems especially increases possibilities in basidiomycetes, since they naturally have a low yield of homologous recombination. Advancements in biotechnological research are mainly limited to two organisms, *P. ostreatus* and *G. lucidum*. For these organisms, successful gene knockouts have been created with and without CRISPR-Cas, and CRISPR-Cas is used to create multiple genomic edits in single experiments. Other achievements of CRISPR-Cas in these organisms are the creation of a strain deficient in NHEJ and the development of a marker-free system using an RNP based method. But, as described in the introduction, expanding the knowledge and techniques to applications in other species is a major challenge in this field. For that reason, this review discussed some key studies focussing on the engineering of *L. edodes*. Besides traditional engineering with transformation vectors and Agrobacterium-mediated transformation, CRISPR-Cas is being used to improve marker systems and create gene knockouts. Further studies are necessary to control the different phases of *L. edodes*' life cycle and to enable multiplexing. In conclusion, genetic engineering of basidiomycetes drives towards the sustainable development of these fungi for development of drugs and other useful secondary metabolites, waste degrading microfactories, and building materials.

The complicated genomic structures that make up the genes and gene clusters responsible for the biotechnologically useful properties of basidiomycetes provide future challenges for genetic engineering. Genomics, metabolomics, and multiple omics studies will aid the further development of new tools and strategies [Arshadi et al., 2023]. This review has shown that existing knowledge on bacteria and ascomycetes can be adapted to drive new research on basidiomycetes.

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