

MINI REVIEW

Use of CRISPR-Cas tools to engineer *Trichoderma* species

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Abstract

Given their lignocellulose degradability and biocontrol activities, fungi of the ubiquitously distributed genus *Trichoderma* have multiple industrial and agricultural applications. Genetic manipulation plays a valuable role in tailoring novel engineered strains with enhanced target traits. Nevertheless, as applied to fungi, the classic tools of genetic manipulation tend to be time-consuming and tedious. However, the recent development of the CRISPR-Cas system for gene editing has enabled researchers to achieve genome-wide gene disruptions, gene replacements, and precise editing, and this technology has emerged as a primary focus for novel developments in engineered strains of *Trichoderma*. Here, we provide a brief overview of the traditional approaches to genetic manipulation, the different strategies employed in establishing CRISPR-Cas systems, the utilization of these systems to develop engineered strains of *Trichoderma* for desired applications, and the future trends in biotechnology.

INTRODUCTION

The genus *Trichoderma* comprises a range of rhizo-competent soil-dwelling moulds with parasitic or saprotrophic lifestyles (Kubicek et al., 2011). They can exploit a diverse range of substrates and thrive on the resources provided by plants, other fungi, and animals. In addition, it has recently been established that members of this genus are widely distributed not only in terrestrial ecosystems but also in marine habitats (Li, Lin et al., 2021; Li, Liu et al., 2021). Given their broad host range and adaptability, species of *Trichoderma* have found widespread industrial and agricultural applications as a

source of enzyme preparations (Zou, Bao et al., 2021; Zou, Li et al., 2021; Zou, Xiao et al., 2021) and biological control agents (Tomico-Cuenca et al., 2021). Moreover, they are important renewable natural resources with high economic value and application potential because of their ability to grow and reproduce on inexpensive substrates, including agricultural and forestry wastes (Zain Ul Arifeen et al., 2019).

Designated as a generally recognized as safe (GRAS) organism by the U.S. Food and Drug Administration, *T. reesei* has been used as a model strain in the cellulase preparation industry for over half a century (Bischof et al., 2016). Some *Trichoderma* fungi,

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including *T. harzianum*, *T. virens*, and *T. longibrachiatum*, have also been used for the production of extracellular enzymes (Dong et al., 2022; Papzan et al., 2021; Zeng et al., 2016). Intriguingly, a commercial enzyme preparation called Lysing Enzymes from *T. harzianum* (Sigma®, L1412) has been commonly used for the preparation of fungal protoplasts (Zeng et al., 2016). *Trichoderma* spp., has been exploited as a biological plant protection agent for nearly a century, which is another important application. The most effective biocontrol properties were mainly attributed to *T. virens* (de Souza Maia Filho et al., 2017), *T. harzianum* (Zhang et al., 2016), *T. koningii* (Gajera et al., 2016), *T. pseudokoningii* (Zavala-Gonzalez et al., 2016), *T. longibrachiatum* (Sridharan et al., 2021), *T. asperellum* (Xian et al., 2020), and *T. viride* (Kumar et al., 2021).

In recent years, a range of *Trichoderma* applications has undergone considerable expansion, facilitated by rapid ongoing developments in the field of biotechnology (Benitez et al., 2004; Bischof et al., 2016; TariqJaveed et al., 2021; Xie et al., 2021; Zhang et al., 2021). Notable in this regard has been the advances made in genetic modification and the generation of massive amounts of genomic data for this genus, including *T. reesei* (Martinez et al., 2008), *T. atroviride* (Kubicek et al., 2011), *T. virens* (Kubicek et al., 2011), *T. longibrachiatum* (Kubicek et al., 2011), *T. harzianum* (Steindorff et al., 2014), and *T. asperellum* (Druzhinina et al., 2018; Kubicek et al., 2019; Li, Lin et al., 2021; Li, Liu et al., 2021). Industrial, agricultural, and even pharmaceutical biotechnologies are particularly reliant on these tools to meet the increasing demands and augment the number and diversity of *Trichoderma*-derived proteins, metabolites, biomolecules, and chemical products (Mukherjee et al., 2013). These advances are of particular relevance in the case of multicellular organisms such as *Trichoderma*, given that genetic modification based on traditional methods is rarely as straightforward as that in unicellular microorganisms (including bacteria and yeasts), owing to complex cellular differentiation, thick chitinous cell walls, and lack of self-replicating vectors (Jiang et al., 2013).

Among the more recent advances in genetic engineering, the use of artificially engineered nucleases is an effective approach for investigating the function of genes and proteins (Tomico-Cuenca et al., 2021). In *Trichoderma*, these nuclease-based editing tools mainly include clustered regularly interspaced short palindromic repeats (CRISPR) RNA-guided Cas9 (CRISPR-Cas9; de Souza Maia Filho et al., 2017) and transcription activator-like effector nucleases (TALENs; Liu et al., 2015). Compared to TALENs and other editing strategies, CRISPR-Cas gene editing methods are currently the most efficient, convenient, and widely used for genome engineering (Burgess, 2013).

The discovery and manipulation of CRISPR and Cas genes has accelerated recent developments in flexible,

cost-effective genomic engineering toolkits based on the programmable targeting of CRISPR-Cas technologies (Deveau et al., 2010). Researchers have employed CRISPR-Cas to modify the genomes of a diverse range of organisms by introducing double-strand breaks (DSBs; Burgess, 2013), which activate sequence variations (insertions, deletions, and rare substitutions near DNA cleavage sites) conducted by non-homologous end joining (NHEJ); precise sequence alterations conducted by homology-directed repair (HDR) with the endogenous repair pathways (artificial supply of repair template; Horwitz et al., 2015). Using CRISPR-Cas tools, it is possible to simultaneously edit multiple loci, highlighting the potential utility of this technique as an extensible system for versatile genome-wide engineering (Cong et al., 2013; Li et al., 2013; Liu & Fan, 2014). Most applications of the CRISPR-Cas system also have direct utility and relevance with respect to *Trichoderma*, for which CRISPR-Cas can be applied to augment and/or enhance pre-existing genetic engineering platforms (Liu et al., 2015). To date, researchers have constructed CRISPR-Cas tools based on diversification strategies and have applied them to functional gene identification, strain modification, and other fields in *T. reesei* (Bodie et al., 2021; Chai et al., 2022; de Souza Maia Filho et al., 2017; Hao & Su, 2019; Li, Lin et al., 2021; Li, Liu et al., 2021; Liu et al., 2015; Rantasalo et al., 2019; Wu, Chen, Huang, et al., 2020; Wu, Chen, Qiu, et al., 2020; Zou, Bao et al., 2021; Zou, Li et al., 2021; Zou, Xiao et al., 2021). Nevertheless, some *Trichoderma* species, including *T. harzianum* (Vieira et al., 2021), *T. atroviride* (Primerano, 2021), and the unidentified species *Trichoderma* sp. LF328 (Vidgren et al., 2020), have only built CRISPR systems, and the rest have not yet been reported to attempt genome editing. For these *Trichoderma* species, the leading research progress in *T. reesei* can serve as a reference paradigm.

In this short review, we focus on the most recent evolution and applications of CRISPR-Cas-mediated genomic engineering for gene editing and its imminent implications regarding the industrial application of *Trichoderma*, mainly including (i) transformation methods, (ii) Cas nuclease and sgRNA delivery strategies, and (iii) applications of CRISPR-Cas genome editing in *Trichoderma* species.

TRANSFORMATION METHODS

At present, neither the initial proofs of concept nor the practical applications of CRISPR-Cas in fungi, including *Trichoderma*, are completely independent of the traditional tools of genetic manipulation, which are responsible for introducing two essential components of the CRISPR-Cas system: Cas genes/proteins and guide RNAs (gRNAs) (or templates). Consequently, classical genetic transformation technology remains an

essential requirement for the development of CRISPR-Cas tools.

A diverse range of genetic manipulation methods have been applied in engineering *Trichoderma* (Figure 1; Li et al., 2017), the most common of which is the polyethylene glycol (PEG)–mediated transformation of protoplasts (PMT; Figure 1A; Penttilä et al., 1987), *Agrobacterium tumefaciens*-mediated transformation (AMAT; Figure 1B; de Groot et al., 1998), electroporation (Figure 1C; Sanchez-Torres et al., 1994), and biolistic delivery (Figure 1D; Lorito et al., 1993). The transformation rates achieved using these approaches tend to differ depending on the technique applied and the target *Trichoderma* species.

PMT

Transformation of fungi tends to be hampered to varying extents by their multicellular structure and thick cell walls; to overcome these barriers, cocktails of fungal cell wall-degrading enzymes are used to generate protoplasts via the enzymolysis of hyphal cell walls (Figure 1A). Being deprived of its protective cell wall, protoplasts are preserved in a hyperosmotic solution,

generally high concentrations of either sucrose (0.6 M) or sorbitol (1.2 M) with a slightly basic pH value (pH 7.5), to prevent swelling and rupture. During transformation, protoplasts, as receptor cells, are transformed with DNA (or other biomolecules, including circular/linearized plasmids, RNAs, proteins, and ribonucleoproteins) using 25% (w:v) PEG 6000 and 50mM calcium chloride. Given its simplicity, relative rapidity, and high yields, this method has become the technique of choice for the transformation of a number of *Trichoderma* species (Cai et al., 2021; Cardoza et al., 2006; Herrera-Estrella et al., 1990). After optimizing the process, the transformation efficiency reached 200~800 colonies per microgram of DNA in *T. reesei* (Herrera-Estrella et al., 1990).

AMAT

AMAT, as a well-established simple and versatile method, is based on the natural infectivity of *A. tumefaciens* towards plants and the transformation of host genomes using a partial Ti vector (Zeilinger, 2004). This process can be readily harnessed for the genetic manipulation of plants and have been adapted to introduce exogenous

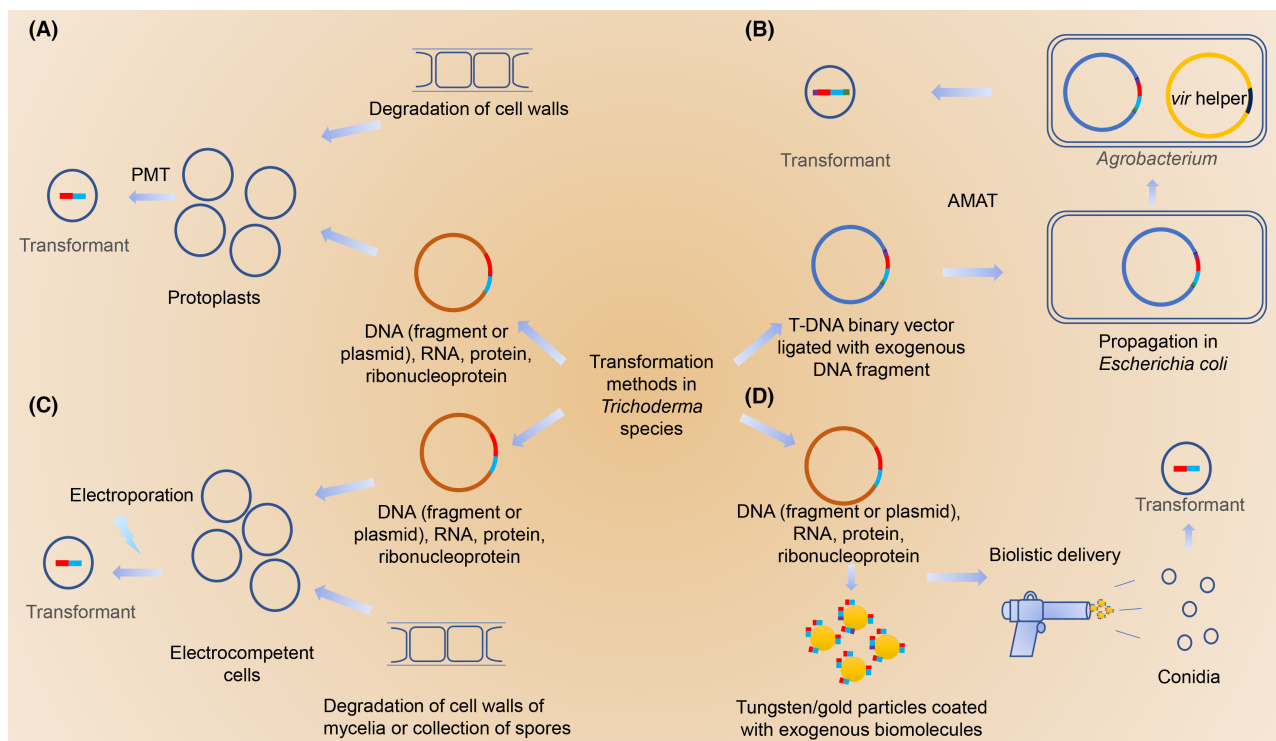


FIGURE 1 Schematic diagrams of classical transformation strategies in *Trichoderma* species. (A) Polyethylene glycol (PEG)–mediated transformation of protoplasts (PMT). (B) *Agrobacterium tumefaciens*-mediated transformation (AMAT). (C) Electroporation. (D) Biolistic delivery. In general, PMT (A), electroporation (C), and biolistic delivery (D) are theoretically compatible with various biomolecules such as linear or circular DNA, RNA, protein, ribonucleoprotein. However, plasmid, ligated with a selectable marker (red) and an expression cassette of a gene of interest (light blue), is the most common biomolecule for transformation. In AMAT (B), the biomolecule is limited to DNA fragments which is flanked by left border (purple) and right border (green). These plasmids all contain kanamycin, ampicillin, or other antibiotic resistance in their skeletons, enabling screening and propagation in *Escherichia coli*.

genetic material in filamentous fungi. Generally, a modified binary vector system is required for the transport of foreign genes into diverse recipient cells (conidia, protoplasts, and even mycelia; [Figure 1B](#)). Using this binary system, the T-DNA and *vir* regions are inserted into two independent plasmids. A selectable marker gene and an exogenous gene of interest are introduced between the T-DNA borders, which are essential for the transformation and release of the DNA fragments therein (Michielse et al., [2004](#)). The released fragments are then randomly inserted (DNA fragments without HDR) or homologously recombined (DNA fragments with HDR) into the genome. The use of this method has been reported with respect to gene disruption in *T. atroviride* (Zeilinger, [2004](#)) and was subsequently successfully developed for the transformation of *T. reesei* using *hph* (encoding hygromycin B phosphotransferase) as the selectable marker gene (Zhong et al., [2007](#)). Furthermore, in 2019, a modified ATMT method using two different *A. tumefaciens* strains was reported that could be used to simultaneously introduce two plasmids in a single step (Wu, Chen, Huang, et al., [2020](#); Wu, Chen, Qiu, et al., [2020](#)). Although AMAT is another common transformation method, it resulted in a lower efficiency of DNA integration and less stable transformants when the ATMT and PMT methods were compared in four different *Trichoderma* species (Cardoza et al., [2006](#)). The inefficiency of AMAT may be due to the fact that the two methods used different receptor cells. In *T. reesei*, AMAT efficiency increased 10- to 50-fold in protoplasts compared to conidia (Zhong et al., [2007](#)).

Electroporation

In this technique, electric current pulses are used to puncture micropores in the cell membrane, thereby enabling foreign DNA to penetrate the membrane and enter the cell. It is essential to select an appropriate field intensity to restore the cell viability. Excessively powerful electric fields can be lethal, owing to irreversible damage to the cell membrane (Li et al., [2017](#)). Electroporation-based genetic manipulation has been established for *T. harzianum* (Goldman et al., [1990](#)). The process of producing competent cells suitable for electroporation was similar to that used to generate protoplasts. Competent cells were primed for DNA penetration using an electric pulse with or without PEG 6000 (optional; [Figure 1C](#); Goldman et al., [1990](#), Cai et al., [2021](#)). Conidial spores have also been used for receptor cell electroporation (Kim & Miasnikov, [2013](#)). Perhaps, it is the most promising protocol for delivering DNA to *Trichoderma* fungi because of its ease, efficiency, and reduced hands-on time. However, there is a lack of clarity regarding the variables of spore electroporation and which conditions are likely to achieve the highest transformation efficiency (Kim & Miasnikov, [2013](#)).

Biolistic bombardment

In biolistic bombardment of cells, DNA-coated gold or tungsten particles are fired into the cells at a high speed ([Figure 1D](#)). It is a rapid and convenient method that does not require the preparation of osmotic pressure-sensitive protoplasts or time-consuming co-culture with *A. tumefaciens*; however, it requires the initial purchase of additional expensive equipment and reagents (Li et al., [2017](#)). To date, biolistic bombardment has been adapted for the transformation of a number of *Trichoderma* species, including *T. harzianum* (Lorito et al., [1993](#)), *T. longibrachiatum*, and *T. reesei* (Hazell et al., [2000](#)), the efficiency of which depends primarily on the following three parameters: scattering distance of particles prior to impacting the cells, vacuum intensity in the cavity, and density and size of particles. Biolistic transformations in fungi are reportedly less efficient than protoplast uptake (Hazell et al., [2000](#)). The transformation efficiency reached only 35–40 colonies per microgram of DNA (linear or circular plasmid DNA) in *T. reesei* (Te'o et al., [2002](#)).

In summary, in *Trichoderma*, PMT is simple, efficient, does not require complicated equipment, and is suitable for a variety of biomolecules. This has become the most common transformation strategy for delivering the CRISPR system ([Table 1](#)).

Cas nuclease and gRNA delivery strategies

The different transformation methods developed for *Trichoderma* can be modified to transform cells with different biomolecules, and a diverse range of strategies have been adopted to introduce Cas nucleases and guide RNAs (gRNAs) into *Trichoderma* ([Table 1](#)). Here, we summarize three common strategies employed for the delivery of Cas nuclease and gRNA: Cas9 in vivo and gRNA in vitro (Cas9-expressing chassis with gRNA in vitro; [Figure 2A](#)), both Cas and gRNA in vivo (plasmid-based CRISPR-Cas; [Figure 2B](#)), and both Cas9 and gRNA in vitro [ribonucleoprotein (RNP)-based CRISPR-Cas] ([Figure 2C](#)).

These three strategies will be discussed in detail below.

Cas-expressing chassis with gRNA in vitro

Trichoderma genes show a high GC bias at the codon wobble position (<http://www.kazusa.or.jp/codon/>). Unlike *Saccharomyces cerevisiae*, the codon optimized *cas9* gene for human cells does not function in many *Trichoderma* fungi (Liu et al., [2015](#); Tomico-Cuenca et al., [2021](#); Zou & Zhou, [2021](#)). Thus, the initial strategy used to establish the CRISPR-Cas

TABLE 1 CRISPR-Cas systems established in *Trichoderma* species

Strategies						
Species	Cas	gRNA	Transformation method	Editing type and application	Efficiency	Reference
<i>T. reesei</i>	<i>T. reesei</i> codon optimized	Transcribed in vitro	AMAT (Cas9) and PEG (gRNA)	Single/multiple gene disruption or replacement;	4.2% (triplex) –100% (single)	Liu et al. (2015)
<i>T. reesei</i>	<i>T. reesei</i> codon optimized	Transcribed in vitro	PMT	Single-gene replacement; gene function investigation	N/A	Liu, Chen et al. (2017); Liu, Wang et al. (2017)
<i>T. reesei</i>	RNP	RNP	PMT	Single-gene disruption	3.5% (<i>cel3c</i>) –14.8% (<i>cbh1</i>)	Hao and Su (2019)
<i>T. reesei</i>	RNP	RNP	PMT	Single/triple gene replacement; chassis modification; strain engineering	6% (double) –23% (single); 12% (triple)	Rantasalo et al. (2019)
<i>T. reesei</i>	<i>T. reesei</i> codon optimized	U6 snRNA promoter	AMAT	Single-gene disruption	1–10%	Wu, Chen, Huang, et al. (2020); Wu, Chen, Qiu, et al. (2020)
<i>Trichoderma</i> sp.	RNP	RNP	PMT	Single-gene replacement; strain engineering	100%	Vidgren et al. (2020)
<i>T. reesei</i>	RNP	RNP	PMT	Single/multiple gene disruption or replacement; marker-free gene disruption	7.4% (marker free) –100% (single); 10.0% (triple)	Zou, Bao et al. (2021); Zou, Li et al. (2021); Zou, Xiao et al. (2021)
<i>T. reesei</i>	<i>Aspergillus niger</i> codon optimized	5S rRNA promoter	PMT	Single-gene disruption	6.7% (heterologous 5S rRNA promoter) –36.7% (native 5S rRNA promoter)	Wang et al. (2021)
<i>T. reesei</i>	RNP	RNP	PMT	Single-gene replacement; marker-recycled iterative replacement; chassis modification	N/A	Chai et al. (2022)
<i>T. reesei</i>	<i>Aspergillus niger</i> codon optimized	5S rRNA promoter	PMT	Single-gene disruption	6.7% (heterologous 5S rRNA promoter) –36.7% (native 5S rRNA promoter)	Wang et al. (2021)
<i>T. reesei</i>	<i>T. reesei</i> codon optimized	U6 snRNA promoter with 1st intron	PMT	Single-gene replacement; gene function investigation	N/A	Bodie et al. (2021)
<i>T. harzianum</i>	<i>T. harzianum</i> codon optimized	<i>T. reesei</i> derived Pol II <i>tef1</i> promoter	Biolistic transformation	Single-gene disruption	N/A	Vieira et al. (2021)
<i>T. atroviride</i>	RNP	RNP	PMT	Single/double gene disruption	N/A	Primerano (2021)

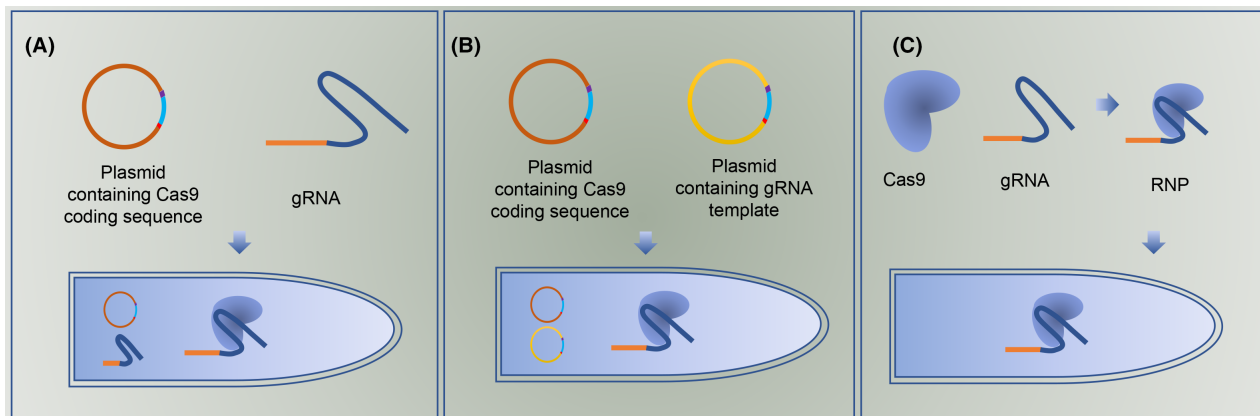


FIGURE 2 Schematic diagrams of CRISPR-Cas systems in *Trichoderma* species. (A) Cas9-expressing chassis with gRNA in vitro. Cas9-expression cassette containing the codon-optimized *cas9* gene with NLS (light blue) is controlled by an appropriate promoter (red) and terminator (purple). gRNA (yellow: crRNA spacer sequence; sapphire: tracrRNA) is transcribed using T7 promoter in vitro and then is transformed with Cas9-expressing chassis cells. (B) Plasmid-based CRISPR-Cas. Cas9-expression cassette and gRNA-transcription cassette can be constructed in a plasmid or separately in two plasmids. (C) RNP-based CRISPR-Cas. RNP is pre-assembled with Cas9 and gRNA in vitro and transformed within cells.

system in *T. reesei* involved the expression of codon-optimized Cas9 nuclease (*Streptococcus pyogenes*) in vivo (Liu et al., 2015, Zou & Zhou, 2021; Table 1). Transcription of gRNA (10–50 µg of gRNA for 10⁶ protoplasts) using the T7 promoter in vitro (Figure 2A) was also the earliest established CRISPR-Cas genome editing platform used for filamentous fungi and has been extensively applied in the engineering of a range of fungi (Chen et al., 2017, Zheng et al., 2017), including the basidiomycete fungus *Ganoderma lucidum* in which the in vitro-transcribed gRNA is introduced into protoplasts expressing the Cas nuclease (cellular host used as a recipient for further engineering) via PEG-mediated transformation (Table 1; Qin et al., 2017). Depending on the fungal genomic sequence information, genome engineering, including multiplexing mutations and knockin/knockout, can be implemented with high efficiency [4.2% (triple loci) ~100% (single locus)] by modifying a 20-bp protospacer of gRNAs corresponding to a target gene sequence (Liu et al., 2015). Consequently, this optimized CRISPR-Cas strategy can save time and labor without the need to identify suitable RNA promoters for gRNA transcripts or repeatedly transform the Cas-expressing plasmid. Moreover, compared with the continuous in vivo transcription of gRNA, transiently transforming gRNAs reduces the risk of off-target modification (Liu et al., 2015).

Plasmid-based CRISPR-Cas

This is a conventional strategy that requires the construction of plasmids for Cas expression and gRNA transcription (Figure 2B). In this approach, Cas expression boxes (codon-optimized *cas* gene with nuclear localization signal sequence [NLS] controlled

by an appropriate promoter and terminator) are either integrated into the genome or self-replicating plasmids (Schuster & Kahmann, 2019). RNA polymerase III promoters, such as SNR52 and U6 snRNA promoters, have been used to transcribe gRNAs in fungi (DiCarlo et al., 2013; Liu et al., 2019). Although both of these promoters are conserved in eukaryotes, prediction of promoters is difficult using bioinformatics, owing to the diversity of canonical splice sites and branch site motifs (Canzler et al., 2016). Both the 5S rRNA (Wang et al., 2021) and U6 snRNA promoters (Bodie et al., 2021; Wu, Chen, Huang, et al., 2020; Wu, Chen, Qiu, et al., 2020) were able to transcribe gRNA in *T. reesei*. Although RNA polymerase III was initially believed to mediate microRNA transcription, circumstantial evidence suggests that the RNA polymerase II promoter is also responsible for microRNA transcription (Lee et al., 2004). In *T. harzianum*, the promoter *tef1* derived from *T. reesei* has been used to control gRNA transcription (Vieira et al., 2021).

RNP-based CRISPR-Cas

Continuous Cas9 expression in vivo has been reported to cause unfavourable phenotypes such as reduced growth (Enkler et al., 2016) and even lethal effects in some organisms (Jiang et al., 2017). Besides, unexpected off-target events often result due to two major factors when there is long-term presence of Cas and gRNA within cells: less stringent recognition of protospacer adjacent motif flanking the target sequence and tolerance to target DNA-gRNA mismatch (Kang et al., 2022). Therefore, researchers have recently developed a strategy designed around an in vitro pre-assembled Cas-gRNA complex for transient genome editing (Figure 2C; Kim et al., 2014). Using this

approach in *T. reesei* QM9414, a pre-assembled RNP of Cas9 (recombinant Cas9 expressed in *Escherichia coli*) and gRNA (transcribed by the T7 promoter in vitro), co-transformed with the *pyr4* gene (syn. *ura3*, encoding orotidine-5'-phosphate decarboxylase) as a selective marker using PMT, has been used to disrupt the major cellulase gene *cbh1* (14.8%) and *cel3c* (3.5%; Hao & Su, 2019; Table 1). To further enhance editing efficiency, the additional use of the detergent Triton X-100 has been reported to facilitate RNP penetration of the protoplast membrane in *T. reesei* and increase the editing efficiency to 100% for single-gene disruption (Table 1). In the control group without Triton X-100, only half of the correctly edited transformants were obtained (Zou, Bao et al., 2021; Zou, Li et al., 2021; Zou, Xiao et al., 2021). The enhanced CRISPR-Cas9 ribonucleoprotein method has been adapted to a variety of fungi such as *Aspergillus oryzae*, *Cordyceps militaris*, and *Claviceps purpurea* (Yu et al., 2022; Zou, Bao et al., 2021; Zou, Li et al., 2021; Zou, Xiao et al., 2021). Multiplex editing requires the introduction of mixed RNPs within cell via, respectively, designing multiple gRNAs targeting different loci. However, it is usually low in efficiency (10.0% for triple genes) due to the limited receptivity of a fungal cell to exogenous biomolecules (Zou, Bao et al., 2021; Zou, Li et al., 2021; Zou, Xiao et al., 2021; Table 1). This suggests that more (≥ 4 loci) gene editing may require tactical improvements such as employing CRISPR-Cas12a which does not require tracrRNA in crRNA processing and performs much easier in multiplex targeting (Paul & Montoya, 2020). The results obtained using this approach indicate that the direct introduction of an RNP complex into fungal cells is an optimal strategy for rapid, simple, and precise genomic engineering, with considerable potential for multiple applications in functional genomics. Moreover, it can be used to minimize off-target events and cytotoxicity associated with the continuous expression of Cas nuclease in cells (Foster et al., 2018). This strategy also offers a promising gene-engineering approach for completely exogenous DNA-free solutions. Eradication of transgenic integration, DNA fragment insertion, and resistance marker selection in engineered mutants is highly accessible for public acceptance of genome edited organisms (Kanchiswamy, 2016). In conjunction with previously established molecular biology tools, this genome engineering technology represents a potentially powerful approach for the genetic manipulation of *Trichoderma* (Primerano, 2021; Rantasalo et al., 2019) and undoubtedly other fungi, thereby contributing to the progress in fungal studies on strain improvement and functional genomics (Zou, Bao et al., 2021; Zou, Li et al., 2021; Zou, Xiao et al., 2021).

In summary, the editing efficiency of different strategies generally depends on the total amount of Cas9, gRNA, or RNP in the cells (Table 1). Therefore, the promoter is critical for the expression of Cas9 and

transcription of gRNA for the in vivo strategy (Table 1). In *T. reesei* C30-cc (Cas9-expressing chassis with inducible promoter *pcbh1*), gene editing was conditionally implemented by inducers (lactose or cellulose) or repressors (glucose; Liu et al., 2015). Similarly, the heterologous 5S rRNA promoter of *A. niger* showed only 6.7%, whereas the native promoter increased the editing efficiency to 36.7% in *T. reesei* (Wang et al., 2021). In addition to the dosage of RNP affecting editing efficiency, Triton X-100 dramatically increased the number of edited mutants using the RNP transformation procedure, which could be attributed to the greatly improved efficiency (3.33-fold) of RNP penetration by improving cell membrane permeability (Zou, Bao et al., 2021; Zou, Li et al., 2021; Zou, Xiao et al., 2021). The activity of two major pathways for the repair of Cas9-induced DSBs is another important factor that affects editing efficiency (Rantasalo et al., 2019). The *T. reesei* strain containing the *mus53* deletion (increased the rate of HDR by suppressing NHEJ pathway) exhibited higher efficiency (12% for triple genes) in multiplexed editing (Rantasalo et al., 2019).

Although all the currently reported CRISPR-Cas systems are based on Cas9 in *Trichoderma*, other Cas nucleases with diverse PAM motifs are probably compatible with *Trichoderma* species (Paul & Montoya, 2020). CRISPR nucleases with longer PAM are expected to cause fewer off-target events. However, due to more stringent requirements of PAM sequences, it will reduce the number of practicable target sites (Kaya et al., 2016). The recent development of prime editing and base editor provides more potential strategies for engineering *Trichoderma* genome (Anzalone et al., 2019; Zhang et al., 2022).

APPLICATIONS OF CRISPR-CAS GENOME EDITING IN *Trichoderma* SPECIES

Non-selectable marker-dependent genome manipulation

To create auxotrophic strains for future experiments, the first genes selected by research groups, mostly focusing on *Trichoderma* species, including *T. reesei* (Liu et al., 2015), *T. harzianum* (Vieira et al., 2021), and *T. atroviride* (Primerano, 2021), were the bidirectionally selectable *ura3* and *ura5*, which encode orotidine 5'-phosphate decarboxylase (URA3) and orotate phosphoribosyl transferase (URA5) (Berges & Barreau, 1991; Table 1). Deletion or mutation of these genes disrupts the pyrimidine biosynthesis pathway, yielding uridine auxotrophic strains (Figure 3). In prototrophic strains, URA5 metabolizes 5-fluoroorotic acid (5-FOA), generating 5' fluorouridine monophosphate, a "suicide" substrate that severely limits cell growth;

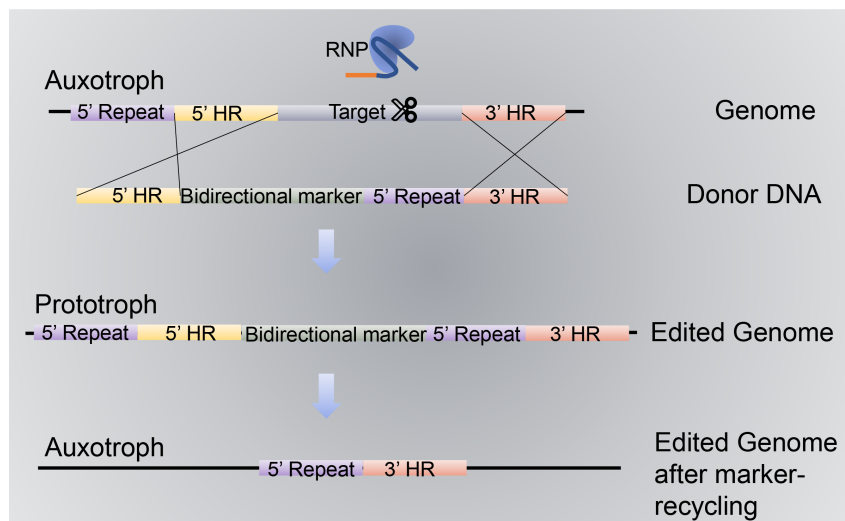


FIGURE 3 Schematic diagram of marker-recycling based on uracil auxotrophy. The optimized deletion construct included a bidirectional marker (e.g. *ura5*) (celadon), the 5' (yellow) and 3' (pink) flanking region of the target gene (grey), and the 5' direct repeat (purple), that is, the upstream sequence of the 5' flanking region. The donor DNA together with the in vitro preassembled RNP was transformed into the auxotrophic strain synchronously to generate edited prototrophic transformants. The bidirectional marker on the transformant genome was recycled via homologous recombination between two direct repeat fragments after incubation on screening plates (e.g. under selection of 5-FOA). By contrast, transformants with ectopic integration of donor DNA were unable to recycle the bidirectional marker due to a shortage of the essential 5' direct repeat for homologous recombination.

URA3, which catalyses the second step in the pyrimidine biosynthesis pathway, can also catalyse 5-FOA to yield the toxic 5' fluorouridine monophosphate (Berges & Barreau, 1991). Using an RNP-based CRISPR-Cas system, auxotrophic strains can be generated without the introduction of exogenous DNA, and it is also possible to edit other loci in the genome by re-complementing native *ura3/ura5* in *T. reesei* (Rantasalo et al., 2019; Zou, Bao et al., 2021; Zou, Li et al., 2021; Zou, Xiao et al., 2021). In addition, the RNP system enabled direct editing of *Trichoderma* strains in the absence of selective pressure; however, the proportion of correctly edited strains remained low (7.37%; Zou, Bao et al., 2021; Zou, Li et al., 2021; Zou, Xiao et al., 2021; Table 1). Importantly, given that these techniques do not involve the transfer of genetic material among organisms, they are not constrained by restrictive GMO-related regulations, which will be a significant factor in gaining public acceptance of this new biotechnology. However, this is impossible with traditional genetic manipulation techniques.

RATIONAL DESIGN OF *Trichoderma* FOR INDUSTRIAL PROPERTY OPTIMIZATION

The “working principle” of CRISPR-Cas will facilitate the genomic engineering of a range of industrially important fungal strains, which will be particularly beneficial, given the paucity of appropriate selective markers and low rates of homologous recombination (Stovicek et al., 2015). In this regard, *T. reesei*

is considered a workhorse for industrial cellulase production (Schmoll, 2008), for which CRISPR-Cas-based multiplexing genome engineering has been adopted to design a hyper-producer via direct modification based on the deletion of repressors, overexpression of activators, and introduction of heterologous enzymes with excellent activity (Fonseca et al., 2020; Liu et al., 2015). For example, in the industrially exploited strain *T. reesei* Rut-C30 (Peterson & Nevalainen, 2012), CRISPR-Cas was used to engineer the following six genetic modifications: overexpression of the activator XYR1, heterologous β -glucosidase CEL3A, heterologous invertase SUC1, and deletion of the native repressor ACE1 and secreted proteases PEP1 and SLP1 (Fonseca et al., 2020). These modifications were found to significantly enhance the rate of protein secretion in *T. reesei* Rut-C30, augmenting inadequate β -glucosidase production and enhancing sucrose utilization, and alleviates the repressive effects of carbon metabolism. Notably, the modified strain showed enhanced (hemi-)cellulase activity, with 72- and 42-fold increases in β -glucosidase and xylanase production, respectively (Fonseca et al., 2020). Given the notable synthesis and secretion characteristics of *T. reesei*, suitably modified strains of this fungus are believed to have considerable promise for the production of large amounts of high value-added protein. Multiplexed CRISPR-Cas in combination with a synthetic expression system (SES) enabled the accelerated construction of *T. reesei* strains and increased the production of fully functional calB to 4 g/L without native background

enzymes (Rantasalo et al., 2019). In another study, 11 native genes (10 secreted lignocellulose-degrading enzymes and one protease activator) were selectively deleted using HDR-stimulated iterative marker recycling (Figure 3), yielding an optimized *T. reesei* chassis that substantially enhanced the production of heterologous genes derived from different organisms (Chai et al., 2022). CRISPR-Cas makes rational design of strains of *Trichoderma* more feasible and convenient than traditional genetic techniques. However, no progress has been made in the design of strains for agricultural applications.

Validation of gene (cluster) function

Over the past two decades, new developments in DNA sequencing have enabled the identification of numerous *Trichoderma* genomic sequences. However, the function of the proteins encoded by these sequences or biosynthetic gene clusters of bioactive substances remains to be established (Rush et al., 2021). Even in the most extensively studied *T. reesei*, poor inherent homologous recombination efficiency currently represents a bottleneck constraining advances in functional genomics (Derntl et al., 2016). Fortunately, the CRISPR-Cas9 system contributes to rapid gene function verification based on bioinformatic annotation. For example, regulation of the putative *TrVib1* protein, an ortholog of *Neurospora crassa* *NcVib1*, was rapidly verified in *T. reesei* using the CRISPR-Cas system (Liu et al., 2015). Similarly, the combined application of bioinformatics analysis and the CRISPR-Cas system has contributed to the characterization of a novel specific transcription factor for GH11 xylanase genes (Liu, Chen et al., 2017; Liu, Wang et al., 2017). CRISPR-Cas was also employed to investigate the causative mutations involved in the reduced viscosity and enhanced volumetric productivity of *T. reesei* mutants with improved industrial fermentation characteristics (Bodie et al., 2021).

Recently, researchers have begun to focus on *Trichoderma* species isolated from specific habitats, which are abundant but rarely investigated fungal sources to produce a wide range of natural products with diverse bioactivities (Liu, Song et al., 2022; Liu, Xu et al., 2022). Moreover, relatively little is known regarding the nature of biosynthetic gene clusters. Thus, the CRISPR-Cas system would appear to be an ideal platform for mining novel natural products associated with cryptic and uncharacterized biosynthetic gene clusters from these newly isolated *Trichoderma* species (Wang et al., 2022).

CRISPR-based regulation in *Trichoderma*

The development of the CRISPR-Cas system will provide a novel approach for elucidating the mechanisms

underlying gene regulation, with biotechnological applications in multiple fields. In addition to investigating the aforementioned functions of transcriptional regulators, CRISPR-Cas9 offers the prospect of precise gene regulation among the newly developed tools, CRISPR activation (CRISPRa) and CRISPR interference (CRISPRi). In this context, dCas9 (inactive nuclease-dead mutant) with a gRNA targeting a promoter region has been demonstrated to down-regulate the expression level of downstream genes, whereas in *Candida albicans*, fusion of dCas to an Mxi1 repressor domain has been found to enhance transcriptional repression (Wensing et al., 2019). In *Pichia pastoris*, CRISPRa/CRISPRi technology has facilitated the more precise regulation of gene expression. Furthermore, when used in conjunction with synthetic promoters in *P. pastoris*, CRISPRa-based gene expression represents a novel “plug-and-play” platform that can be applied to produce customized hosts with high-level expression that responds to defined signals (Liu, Song et al., 2022; Liu, Xu et al., 2022). However, CRISPRa/CRISPRi systems are yet to be developed for editing the *Trichoderma* genome. It is important to enable partial loss of gene function via precise and quantitative activation/repression, rather than complete loss of gene function. Thus, CRISPRa/CRISPRi is a promising technology for future application in *Trichoderma*.

FUTURE PERSPECTIVES AND CONCLUSIONS

Trichoderma spp. are well-studied model fungal organisms because of their powerful cellulase productivity and biocontrol properties. Their products include industrial enzyme preparations used in pulp, biofuels, food, feed, and other fields, as well as biofertilizers, biopesticides, and bioremediation agents. In addition to these major applications of *Trichoderma* species, the fields of sustainable green and white biotechnology have become increasingly important for the environmentally safe production of humanized proteins, antibiotics, and other bioactive natural products. Although a number of relevant key factors to improve strain properties have been discovered in recent decades, traditional genetic manipulation has become a bottleneck for further biotechnological exploration of *Trichoderma*.

CRISPR-Cas genome editing technology enables genetic engineering of a variety of organisms and offers numerous hitherto unattainable strategies for genetic manipulation. Gene-edited crops and mushrooms have been approved for marketing by several countries and organizations. Notably, the legalization of CRISPR-Cas-edited products was realized in less than 5 years following the initial establishment of the

CRISPR-Cas9 concept, thereby highlighting the public's willingness to accept the safety of the technology. Given its appropriate iterative strategy, ease of development, and broad applicability, the CRISPR-Cas system can undoubtedly be applied in a wide range of biotechnological fields. The newly discovered applications of CRISPR-Cas in fungal genome editing have unique and powerful capabilities and potential biotechnological applications. Coupled with its efficiency and simplicity, this system will also be broadly applicable in modifying the genomes of little studied and newly isolated strains of *Trichoderma*, and considering its far-reaching scope, CRISPR-Cas genome engineering will inevitably expand the application of *Trichoderma* in the fields of industry, agriculture, medicine, and food.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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