Review Article

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A Molecular Genetics Approach to Elucidating the Mechanisms Underlying Lignin Degradation by White-Rot Fungi

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Abstract
White-rot fungi play important roles in the global carbon cycle by efficiently degrading lignin and polysaccharides from lignocellulose. Over the years, extensive efforts have been made to elucidate the mechanisms underlying lignin degradation by white-rot fungi. One of them is a molecular genetics approach, which includes genetic modification to alter wood-degrading abilities or metabolism, with the aim of producing commercially valuable chemicals from unutilized lignocellulosic resources. Molecular genetic studies have been conducted on several species, including *Pleurotus ostreatus*, *Phanerochaete sordida*, and *Phlebia* sp., for which a genetic transformation system has been developed. However, the techniques and methodologies available for these fungi are limited, posing a serious bottleneck. Here, we describe recent studies that have developed powerful and effective techniques and methodologies, removing the restrictions of molecular genetics studies on lignin degradation by white-rot fungi.
Keywords: *Lignin degradation*, *Lignin modifying enzymes*, *Wood decay*

INTRODUCTION

The major lignocellulosic components, polysaccharides (cellulose and hemicelluloses) and aromatic heteropolymers (lignin), are considered promising resources for sustainable biorefinery industries. To develop efficient and eco-friendly processing methods for converting natural polymers into value-added chemical products, various enzymes involved in the depolymerization and bioconversion of each of the major components have been isolated from bacteria and fungi, and characterized extensively [1–4]. However, most microorganisms cannot efficiently decompose or degrade lignocellulose, especially lignin, in wood biomass because of its complexity [5–7]. White-rot fungi efficiently decompose lignin and polysaccharides in wood biomass. Therefore, understanding the mechanisms underlying lignin degradation by white-rot fungi would contribute to the development of effective methods for delignification or pretreatment, leading to the utilization of the resultant polysaccharides as sustainable alternatives to fossil resources. Lignin-modifying enzymes produced exclusively by white-rot fungi, lignin peroxidases (LiPs), manganese peroxidases (MnPs), and versatile peroxidases (VPs) are considered key enzymes for lignin degradation based on *in vitro* studies/reactions [8–13]. White-rot fungi can also efficiently transform and mineralize persistent organic pollutants [14–17], a function related to their lignin-degrading mechanisms. Therefore, white-rot fungi can be used for the bioremediation of environmental pollutants, as well as for the pretreatment of lignocellulose in biorefineries.

One effective approach for elucidating and modifying lignin-degrading mechanisms/abilities is molecular genetics, which is roughly divided into two approaches: forward and reverse genetics (Fig. 1). The reverse genetics approach has been used for several white-rot fungi, such as *Pleurotus ostreatus*, *Phanerochaete sordida*, and *Phlebia* sp., for which a genetic transformation system has been developed

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[18–20]. However, both molecular genetics approaches have been constrained by the limitations of available experimental tools. Here, we describe recent molecular genetic studies, especially those focusing on techniques and methodologies that have recently been established for white-rot fungi.

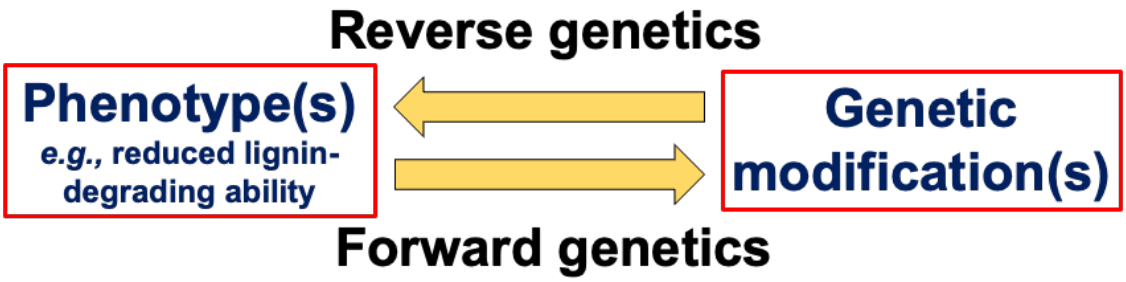

Fig. 1. Simple schematic representation showing different two genetic approaches: forward and reverse genetic approaches.

Gene-targeting by homologous recombination

Reverse genetics analyzes the effects of genetic modifications of interest (Fig. 1). For example, overexpression or inactivation of gene(s) encoding enzyme(s) or regulator(s) related to lignin/lignocellulose degradation [21,22]. Plasmid(s) containing cassette(s) for overexpression or RNA interference (RNAi) are frequently introduced into white-rot fungi [23–25]. However, the introduced exogenous DNA(s) are randomly integrated into the host chromosome(s). Partially deleted DNA fragments are also frequently integrated [18, 26] (Fig. 2). Therefore, unintended gene disruption by random integration may occur, and the degree of expression or inactivation may also differ among transformants because the number of the integrated cassette(s)/plasmid(s) may vary. These differences result in variable phenotypes among the transformants. Therefore, it is difficult to determine the precise effects of these genetic modifications.

Homologous recombination (Fig. 2) is frequently used for precise reverse genetics in various fungi. In this case, DNA fragment harboring two flanking homology arms (5′- and 3′- ones) is introduced (Fig. 2). This allows the introduction or replacement of a single cassette at the target site, thereby making it easier to examine the effects of genetic modification. Homologous recombination is sometimes used as a gene-targeting technique to distinguish random integrations. Gene deletion by homologous recombination has been reported in white-rot fungi such as *Phlebia* sp. [26]. However, the frequency was very low in the wild-type strains [27–29], and the introduced exogenous DNA(s) are randomly integrated in most cases even if they harbor the homology arms. This is probably because the non-homologous end-joining (NHEJ) pathway is dominant in white-rot fungi (Fig. 2). Previously, it was found that disruption of the NHEJ-related genes *ku70*, *ku80*, or *lig4*, results in a significant increase in the frequency of homologous recombination in the ascomycetous filamentous fungus *Neurospora crassa* [30,31]. Following this finding, high-frequency gene targeting was established in the white-rot fungus *Pleurotus ostreatus* by *ku80* deletion [29]. Strain 20b, a *ku80* deletant from the monokaryotic *P. ostreatus* strain PC9, is now frequently used in studies on lignocellulose degradation by this species. For example, the effects of single-gene deletion of lignin-degrading enzyme-encoding genes, *vp1*, *vp2*, *mnp2*, or *mnp3* (formerly *mnp4*, *mnp2, mnp9*, or *mnp3*, respectively) were characterized [29,32,33]. The

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effects of defects in peroxisome biogenesis (*pex1* deletion) on lignin-degrading abilities and the utilization of carbon sources have also been analyzed [34]. Recently, the effects of histone modification on the transcriptional expression of wood-degrading enzyme-encoding genes were analyzed using *ccl1* deletants [35]. However, this methodology may not be applicable to various other white-rot fungi, as it requires the generation of *ku* or *lig4* deletant from the wild-type strain by homologous recombination. Considering the low frequency of homologous recombination, it is necessary to generate many genetic transformants and screen them for the gene deletants. To this end, developing efficient genetic transformation system is required for each species, which is challenging and time-consuming.

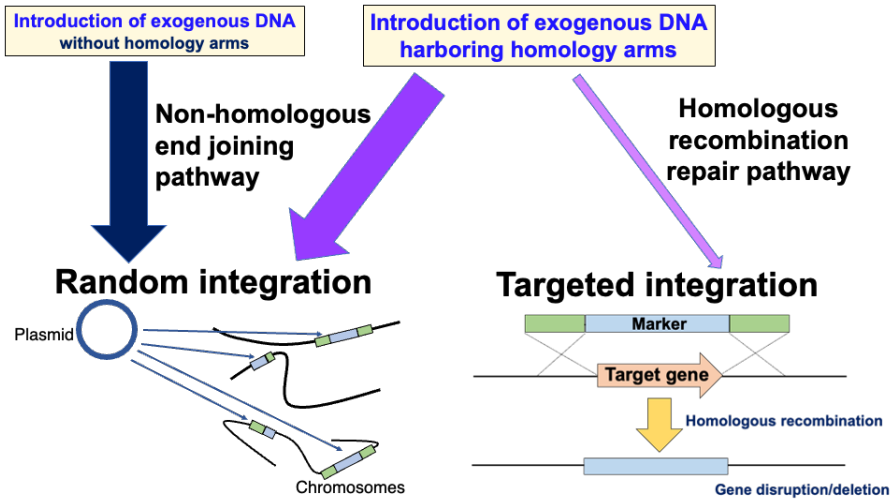

Fig. 2. Two major pathways integrating exogenous DNA into the host chromosomes when transformation.

CRISPR/Cas9 in white-rot fungi

Another gene-targeting method is the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (CRISPR/Cas9)-assisted gene mutagenesis, which is an adaptive immune system found in archaea and bacteria [36–38] that has recently been utilized as a versatile gene-targeting tool. The Cas9 endonuclease is guided to a targeted chromosomal site by a 20-bp single guide RNA (sgRNA), which results in the cleavage of genomic DNA at a specific site on the chromosome, followed by NHEJ-mediated repair. This sometimes introduces mutations at the target site due to errors in the repair process.

Strategies for CRISPR/Cas9-assisted gene mutagenesis are broadly divided into two categories: DNA-based and pre-assembled Cas9 ribonucleoprotein (RNP)-based methods (Fig. 3). The former introduces exogenous DNA-containing expression cassettes for both sgRNA and Cas9 via polyethylene glycol/$CaCl_2$- or Agrobacterium-mediated transformations. The latter introduces Cas9/sgRNA, which was pre-assembled *in vitro*. DNA-based methods can be used if genetic transformations are available; therefore, unlike high-frequency homologous recombination, this method can be applied to various white-rot fungal species. It was recently reported in many white-rot fungi such as *P. ostreatus*, *Ganoderma lucidum*, *Lentinula edodes*, and *Gelatoporia* (formerly *Ceriporiopsis*) *subvermispora* [39–43]. RNP-based methods can be applied to more various white-rot fungi as this method can be performed as

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long as protoplasts can be generated efficiently. At this moment, RNP-based methods have been reported for some white-rot fungi, including *P. ostreatus* and *Dichomitus squalens* [44,45].

CRISPR/Cas9-assisted gene mutagenesis has been reported in many white-rot fungi. However, almost all reports have targeted *pyrG*/*ura3*, a gene encoding orotidine 5′-phosphate decarboxylase, to efficiently screen genome-edited strains by examining 5-fluoroorotic acid resistance. Future studies are required to utilize this technique in functional analyses. Recently, we used a DNA (plasmid)-based method to conduct a functional analysis of genes involved in the sexual development of *P. ostreatus* [46] and lignin degradation by *G. subvermispora* [47]. In these cases, the introduced plasmid(s) were randomly integrated into the host chromosomes. For more precise gene-targeting experiments using DNA-based CRISPR/Cas9, a transient transformation system without the integration of plasmid(s) into the host chromosome should be developed/improved [48].

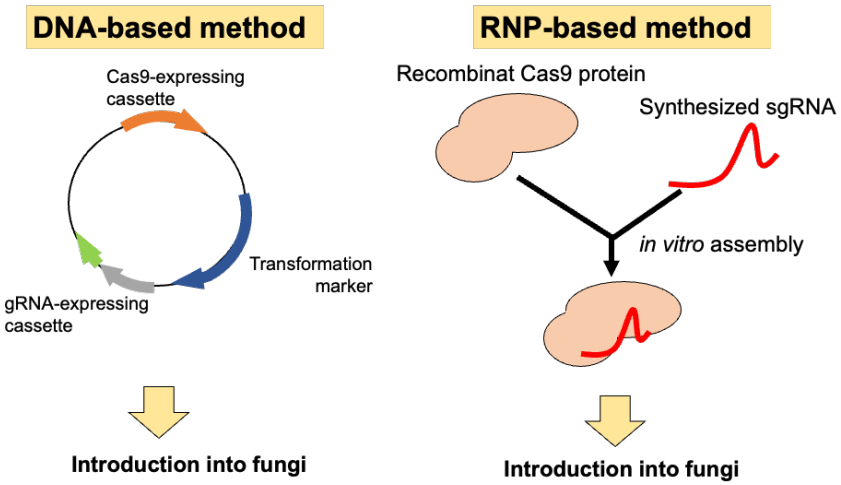

Fig. 3. Schematic representation of two major approach for genome editing using CRISPR/Cas9.

Forward genetics approach in *P. ostreatus*

Forward genetic isolates mutants exhibited a phenotype/trait of interest, followed by the identification of the gene mutation(s) responsible for it. Therefore, unlike reverse genetics, this approach is unbiased. The forward genetic approach was previously used in *Phanerochaete chrysosporium* to isolate mutants in which the production of lignin-modifying enzymes was constitutionally active [49]. However, to the best of our knowledge, the gene responsible for this mutant phenotype is yet to be identified. This may be due to the difficulty and complexity of genetic analysis in *Agaricomycetes* including white-rot fungi, as they generally have multiple mating types [50]. Efficient forward genetics studies in *Agaricomycetes* have been conducted mostly in non-wood-decaying *Coprinopsis cinerea* to investigate the mechanisms underlying fruiting development (*i.e.*, mushroom formation) [51–54]. In this fungus, useful genetic markers and linkage maps as well as well-assembled and annotated genome databases are available [55–57], making it relatively easier to identify the responsible genes. However, substantial efforts are required to develop these tools, and it is challenging to apply the methodologies used in *C. cinerea* to white-rot fungi.

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Accelerated developments in next-generation sequencing technology have allowed whole-genome resequencing to identify mutations and polymorphisms between two strains, such as a mutant and its parental strain. This may facilitate the identification of the responsible mutation(s). However, UV or chemical mutagenesis generates many (over 100) single-nucleotide substitutions in each mutant strain; therefore, methods to narrow down the responsible mutation(s) are important. To this end, we proposed a simple genetic analysis method using conventional PCR based on genome data. Nucleotide polymorphisms between compatible monokaryotic *P. ostreatus* strains, PC9 or PC15, were identified using genome data (at 0.5 Mbp intervals). We designed primer pairs to amplify genomic fragments from either strain (Fig. 4A). This allowed us to easily determine which parent strains, PC9 or PC15, each F_1 progeny inherited each of the genetic loci (Fig. 4B).

Using simple genetic analysis and next-generation sequencing technology, we identified the gene mutations responsible for the reduced lignin-degrading ability of *P. ostreatus* (Fig. 5A). Firstly, mutants defective in decolorization of Orange II or Remazol Brilliant Blue R (RBBR) were generated from PC9 by UV mutagenesis. Most of the mutants degraded less lignin in beech wood sawdust medium supplemented with wheat bran than PC9 did [34,58–60]. We then identified the genetic marker(s) that were strongly linked to the mutant phenotypes (*e.g.*, defects in decolorization) among the F_1 progeny from a cross between each mutant and PC15. Gene mutations located close to the linked marker were identified in each mutant using whole-genome resequencing. We performed complementary transformations and/or targeted gene deletions to confirm that each identified gene was responsible for the mutant phenotype. To date, five genes have been identified. The genes *wtr1* and *gat1* encode putative agaricomycete-specific DNA-binding transcription factors, *chd1* and *hir1* putative chromatin-remodeling factors, and *pex1* encodes a peroxisome biogenesis factor (Fig. 5B). Considering that most of the identified genes encode putative transcriptional regulators, the transcriptional regulatory mechanisms of lignin degradation will be elucidated based on forward genetics studies in future [35,61,62].

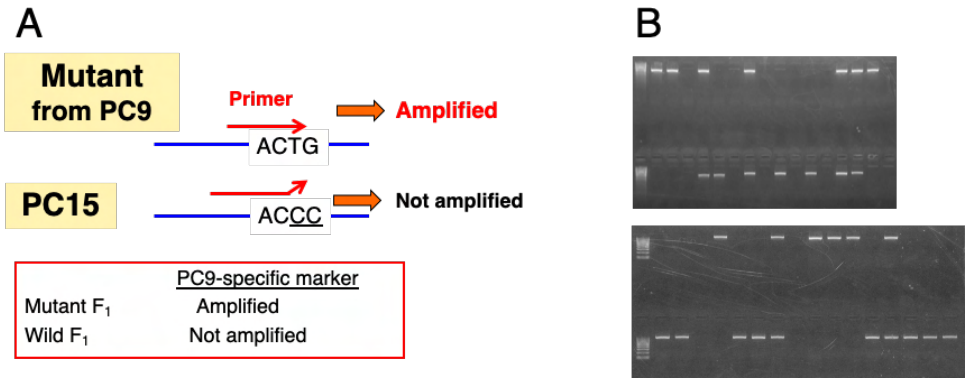

Fig. 4. The proposed method to efficiently narrow down the responsible gene(s) based on genome database. (A) Nucleotide polymorphisms between the two compatible monokaryotic wild-type strains, PC9 and PC15, were identified using the genome database. These differences were determined by PCR to reveal from which parent strains, PC9 or PC15, each F_1 progeny inherited each of the genetic loci. (B) Examples of PCR experiments among F_1 progeny from a cross of PC15 and a mutant derived from PC9.

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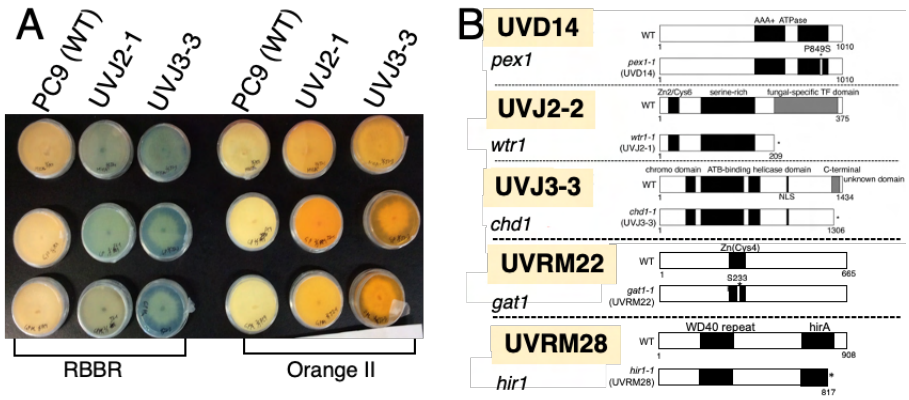

Fig. 5. Identification of genes responsible for OII and RBBR decolorization by *P. ostreatus*. (A) Examples of mutants. This picture is derived from my previous paper [58]. (B) List of mutations responsible for the decolorization that have been identified to date.

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