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REVIEW ARTICLE

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Genetic Manipulation and Transformation Methods for *Aspergillus* spp.

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ABSTRACT

Species of the genus *Aspergillus* have a variety of effects on humans and have been considered industrial cell factories due to their prominent ability for manufacturing several products such as heterologous proteins, secondary metabolites, and organic acids. Scientists are trying to improve fungal strains and re-design metabolic processes through advanced genetic manipulation techniques and gene delivery systems to enhance their industrial efficiency and utility. In this review, we describe the current status of the genetic manipulation techniques and transformation methods for species of the genus *Aspergillus*. The host strains, selective markers, and experimental materials required for the genetic manipulation and fungal transformation are described in detail. Furthermore, the advantages and disadvantages of these techniques are described.

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1. Introduction

Species of the genus *Aspergillus* are widely distributed among natural environments and have several effects on humans [1,2]. More than 300 species have been identified to date, of which several strains also have detrimental or beneficial effects for humans [3]. For example, *Aspergillus fumigatus* and other human pathogenic *Aspergillus* species cause aspergillosis, including invasive aspergillosis, chronic pulmonary aspergillosis, and allergic bronchopulmonary aspergillosis [4–7]. Several fungi (*A. flavus*, *A. parasiticus*, and *A. ochraceus*) infect agricultural crops during the harvest or post-harvest stages, and spoil crops or produce detrimental secondary metabolites in them, called mycotoxins, causing mycotoxin contamination [8–11]. Although *Aspergillus* spp. have detrimental effects on humans, these species are also beneficial for the food and pharmaceutical industries [12–14]. *A. niger* and *A. oryzae* serve as factories that produce the organic acids and enzymes that are required for various industries [12,14]. For the food industry, generally recognized as safe fungi are used in the preparation of traditional fermented foods [15]. Many researchers are investigating the development of novel fungal strains, heterologous expression systems, and novel secondary metabolites through advanced genetic manipulation techniques because of the usefulness of these fungi.

Sequencing analyses of approximately 19 genomes of the species *Aspergillus* were performed, as well as several comparative genome analyses of various strains in the same species, and it proved to be very useful for understanding the function of genes and the species *Aspergillus* [3]. Through genomic analyses, transcriptomic, proteomic, and metabolomic analyses have helped understand fungal biology and find useful genes or metabolic gene clusters [16,17]. Advanced genetic manipulation techniques are being developed and applied to fungal strain improvement and gene utilization in order to understand and use the functions of various useful genes [18,19]. However, unlike *Escherichia coli* or *Saccharomyces cerevisiae*, the species of *Aspergillus* have limited selective markers, low efficiency transformation tools, and constraint of genetic engineering tools. Therefore, advanced genetic manipulation techniques are being developed and applied to fungal strain improvement and gene utilization. Furthermore, genetic research and strain improvement are developing very quickly through genetic engineering techniques such as the clustered regularly-interspaced short palindromic repeats (CRISPR)–Cas system [19,20]. In this review, we present recent information on genetic modification, transformation, and selection techniques, and host strains required for genetic research in species of *Aspergillus*.

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2. Host strains and selective markers

One of the most important items in the entire genetic engineering experiment is the selection of a host strain and a selective marker gene suitable for the genetic manipulation methods. Appropriate host strains and selective markers are essential for reducing the probability of false positive transformants and obtaining a high-yield final product.

2.1. Fungal host strains

Wild type strains can be used for genetic engineering in some fungal species because the genome information is available [21]. However, modified strains are generally used to increase the efficiency of trials and experiments [22]. For example, protease deficient strains are generally used in heterologous protein expression systems [23–25]. The most important item to consider when selecting host strains is which auxotrophic selective marker, except for drug resistant markers, is used in the genetic engineering [26–28]. This is because host strains with specific gene deletions should be used when an auxotrophic selective marker is used in genetic engineering. In most filamentous fungi, the *pyrG*, or *pyrG* ortholog genes, which encode for orotidine-5′-monophosphate decarboxylase, are used as auxotrophic markers; therefore, *pyrG* defect mutants are used for host strains [27,29–31]. Another important consideration is the selection of a host strain with high transformation efficiency. Homologous integration frequency was reportedly increased by *Ku*-deficient mutation in most filamentous fungi [32–36].

2.2. Selective marker genes

In species of *Aspergillus*, 2 selective markers, drug resistance markers, and auxotrophic markers are widely used for genetic engineering experiments [21,37] (Table 1). Drug (antibiotic) resistance genes are generally used as dominant selection markers which are not necessary for special host strains [38]. If one of the *Aspergillus* species is susceptible to an antibiotic, it can be widely used in any species. The most commonly used marker gene is a hygromycin resistance gene, which can be used in most filamentous fungi including *A. fumigatus*, *A. oryzae*, *A. nidulans*, and *A. terreus* [38–40]. However, *A. flavus* NRRL 3357 is resistant to the hygromycin and cannot be used for transformation. Instead of hygromycin, the phleomycin and pyrithiamine resistance genes have been used for *A. flavus* transformation [27]. With these genes, the bleomycin resistance gene has been used for drug resistance markers in other species of *Aspergillus* [27,41]. Although drug resistance markers have the advantage of not needing a specific host strain, their use is limited due to expensive antibiotics and issues with genetically modified organisms.

Auxotrophic genes, defined as genes that encode an essential protein for biosynthesis of an essential nutrient, are widely used for selection of positive transformants for use in genetic engineering [42]. As mentioned above, the *pyrG* gene is widely used as a selective marker for *Aspergillus* transformation [43]. Various *pyrG*-deficient mutants have been developed and these strains cannot grow in media without uridine or uracil, but they can survive by adding uridine or uracil to the medium, so it is easy to use in selection [30,44]. Another important feature of the *pyrG* marker is that it can be developed with a recyclable marker system, so multiple genes can be knocked out [45–47]. Although the *pyrG* gene is widely used in various *Aspergillus* species, other auxotrophic marker genes such as *argB* and *sC* are used only with a few *Aspergillus* species [37]. Several mutant strains were developed as the host strains in *A. nidulans* or *A. oryzae* in order to use the auxotrophic genes. For example, Oakley et al. [33] developed a variety of auxotrophic heterologous markers that can be used in *A. nidulans* selection. In *A. oryzae*, other auxotrophic markers such as *argB*, *niaD*, and *adeA* have been used for selection markers for transformation [37]. If multiple selective markers can be used in a host strain, it is very convenient and efficient to generate multiple deletion mutants or complemented strains [28,48].

3. Methods of fungal transformation

Unlike bacteria or yeast strains, transformation efficiency is low in filamentous fungi, and therefore the transformation process is considered as a bottleneck in genetic engineering [21,49]. Also, species of *Aspergillus* contain a rigid cell wall, which is the main hurdle for increasing transformation efficiency. Currently, several transformation methods have been developed for *Aspergillus* spp., and three methods, including polyethylene glycol (PEG)-mediated transformation (PMT), *Agrobacterium*-mediated transformation (AMT), and electroporation (EP), are mostly used. Only a few studies have been conducted though the biolistic transformation (BT) system in *A. nidulans* and *A. giganteus* [50,51]. Therefore, the process and strategies for these three methods will be discussed in below (Figure 1).

3.1. PEG-mediated transformation

Among the various transformation methods, PMT is the most used method with species of *Aspergillus*. PMT was primarily explored in yeast [52,53]. PMT

Table 1. Lists of certain selective markers and cell wall degrading enzymes used in *Aspergillus* transformation.

Fungus	Cell wall lysing enzymes[a]	Drug resistance markers[b]	Auxotrophic markers[c]
A. aculeatus	Mixture of Yatalase, Lysing enzymes, and Glucanex Mixture of β-D-glucanase and Driselase	*hph*	*pyrG*
A. awamori	Novozyme 234	*hph*	*amdS, pyrG*
A. brasiliensis	Lysing enzymes Mixture of β-D-glucanase and Driselase	*hph*	*pyrG*
A. carbanarious	VinoTaste Pro Mixture of β-D-glucanase and Driselase	*bar, hph*	–
A. delacroxii	–	*hph*	–
A. flavus	Mixture of β-glucuronidase, Driselase, and Lysing enzyme Mixture of β-D-glucanase G, Driselase, cell-wall digesting enzyme, and Lysing enzyme VinoTaste Pro	*ble, ptrA*	*pyrG*
A. fumigatus	Lysing enzyme Mixture of Lysing enzyme and VinoTaste Pro Mixture of Novozyme 234 and β-glucuronidase Mixture of Novozyme 234 and Helicase Mixture of β-D-glucanase and Driselase	*ble, hph ptrA*	*pyrG, argB, niaD*
A. giganteus	Novozyme 234	*hph*	
A. kawachii	Mixture of yatalase and Cellulase R-10	*hph, ptrA*	*sC, argB, pyrG*
A. luchuensis	Mixture of β-D-glucanase and Driselase	*hph*	
A. nidulans	VinoTaste Pro Mixture of Yatalase and Kitalase Mixture of Novozyme 234 and β-D-glucanase Mixture of β-D-glucanase and Driselase	*bar, ble, hph*	*amdS, argB, biA, pyrG, pyroA, riboB, trpC,*
A. niger	Mixture of Lysing enzyme, Snailase, and Lysozyme Mixture of Lysing enzyme and Vinoflow FCE Mixture of Lysing enzyme, Chitinase, and β-glucuronidase Mixture of Novozyme 234 and β-glucuronidase Mixture of β-D-glucanase and Driselase Lysing enzyme	*ble, hph, ptrA*	*adeA, amdS, argB, hisB, nicB, pyrG, sC, trpC,*
A. novofumigatus	Glucanex	*hph*	*pyrG*
A. oryzae	Lysing enzyme Mixture of Novozyme 234 and β-glucuronidase Mixture of Novozyme 234, Hemicellulose, and Celluclast Mixture of Novozyme 234 and Cellulase R-10	*ble, ptrA*	*adeA, amdS, argB, niaD, pyrG, sC*
A. parasiticus	Mixture of β-glucuronidase and Novozyme 234 Mixture of Driselase, Cell-wall digesting enzyme, β-glucuronidase and Lysing enzyme	*ptrA*	*pyrG, niaD*
A. sojae	Lysing enzymes	*ble, ptrA*	*pyrG*
A. terreus	Glucanex	*hph, ptrA*	*pyrG*

[a]Novozyme 234 is no longer available.
[b]*hph* (hygromycin resistance gene; or *hygB*), *bar* (bialaphos resistance gene), *ble* (bleomycin resistance gene), *ptrA* (pyrithiamine resistance gene).
[c]*adeA* (encoding phosphoribosylaminoimidazole-succinocarboxamidesynthase), *amdS* (encoding acetamidase), *argB* (encoding ornithine carbamoyl-transferase), *biA* (encoding putative bifunctional dethiobiotin synthetase), *hisB* (encoding imidazoleglycerol-phosphate dehydratase for histidine biosynthesis), *niaD* (encoding nitrate reductase), *nicB* (encoding nicotinate mononucleotide pyrophosphorylase), *pyrG* (encoding orotidine 5′-monophosphate decarboxylase), *pyroA* (encoding a protein required for biosynthesis of pyridoxine) *riboB* (encoding putative GTP cyclohydrolase for riboflavin biosynthesis), *sC* (encoding ATP sulfurylase), *trpC* (encoding putative indole-3-glycerol-phosphate synthase).

has used in *A. nidulans* for transferring the acetamidase-containing plasmid [54]. The procedure described in this paper was later modified and applied to another species of *Aspergillus*. Then, Szewczyk et al. [55] reported a well-organized PMT protocol, and the methods in this protocol were modified and used according the species.

The PMT procedure simply involves fungal culture, protoplast preparation *via* cell wall degradation, DNA delivery *via* PEG incubation, transformant regeneration, and selection (Figure 1(A)). Protoplasts are cells with the cell walls removed, which are mainly used as recipient cells for PMT [56]. The process of generating protoplasts is a key step in PMT [55]. Germ tubes or young mycelia are mainly used for *Aspergillus* protoplast transformation, treated with various cell wall lysing enzymes dependent on fungal species and cell types. For fungal cell wall lysis, yatalase (from *Corynebacterium spp*; consist of chitinase, chitobiase, and beta-1,3-glucanase; Takara), VinoTaste Pro (from *Trichoderma harzianum* and *A. niger* consist of polygalacturonase and 1,3-β-glucanase; Novozyme), and lysing enzymes (from *T. harzianum*, mixture of β-glucanase, cellulase, protease, and chitinase; Sigma-Aldrich) are mostly used as lysing enzymes, and they are mixed according to the species (Table 1). After protoplast generation, the protoplast solution is mixed with the exogenous DNA (PCR products or plasmid) under a high concentration of PEG (40–50%) and $CaCl_2$ condition which induce DNA uptake and membrane

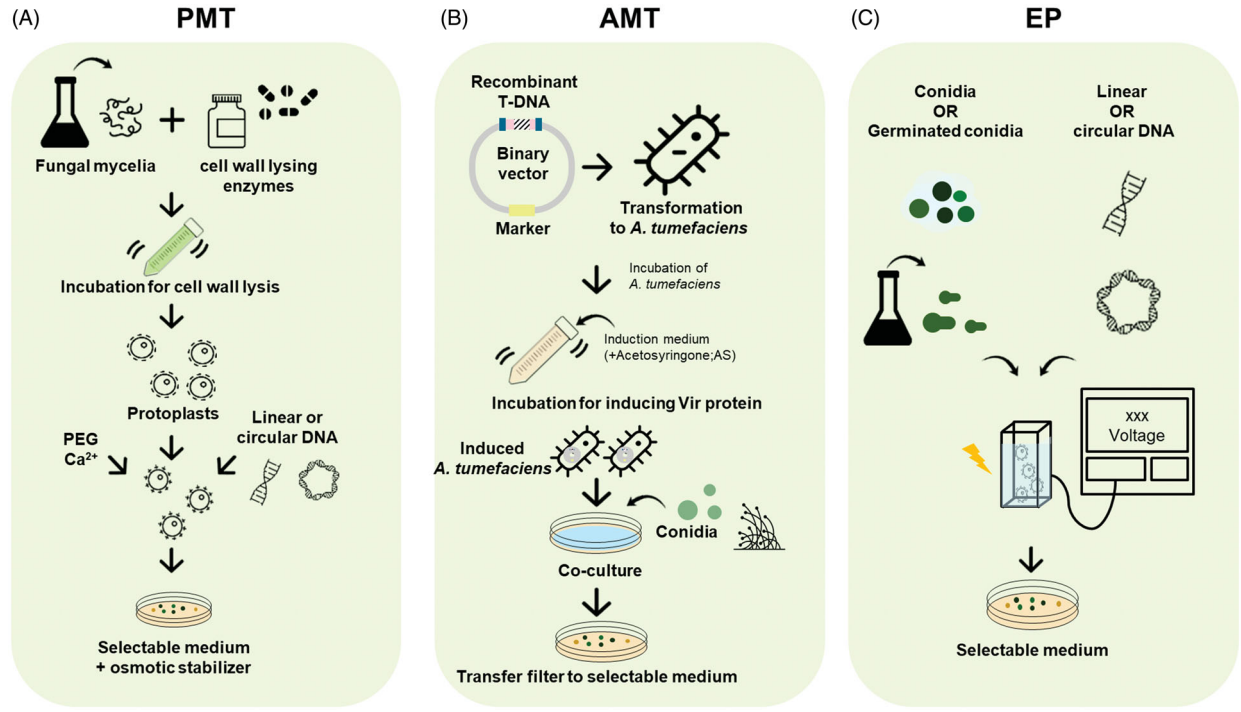

Figure 1. Transformation methods for species of *Aspergillus*. (A) Procedures of PEG-mediated transformation (PMT); (B) *Agrobacterium*-mediated transformation (AMT); and (C) electroporation (EP) methods used in species of *Aspergillus*.

permeability. Protoplasts are sensitive to osmotic stress, so during preparation and regeneration of protoplast osmotic stabilizers, such as potassium chloride and sorbitol, they should be added into the buffer solution and selective media.

Overall, PMT is widely used with many filamentous fungi because it does not require expensive equipment and the procedure of PMT is simple [49,55]. In addition, PMT is not necessary the special vector and bacteria required for AMT. However, in order to achieve success in transformation, culture condition, buffer composition, and cell wall degrading enzymes should be optimized for each species. For some species of *Aspergillus*, including *A. nidulans*, *A. oryzae*, and *A. fumigatus*, the procedure of PMT is well established [37,55,57], but it is essential for optimize an appropriate method, especially lysing enzyme, for other species.

3.2. Agrobacterium-*mediated transformation*

Agrobacterium tumefaciens is a plant pathogenic bacterium which causes crown gall disease in plants [58]. This bacterium contains tumor-inducing plasmid (Ti-plasmid) which can enter the plant cell and insert of small transfer DNA (T-DNA) into the infected plant cell genome. Using this principle, *A. tumefaciens* is used as a vector for gene transfer in various filamentous fungi. AMT was first used for genetic transformation in species of *Aspergillus* in 1998 by de Groot et al. [59]. Until now, AMT has been used for the exogenous DNA introduction in more than 10 fungal species (Table 2). Among these studies, Michielse et al. [63] well described the detailed protocol in *A. awamori*.

The AMT procedure includes construction of binary factor, transformation of *A. tumefaciens* with binary factor, preparation of fungal conidia, *Agrobacterium*-fungal cocultivation, and transformation selection (Figure 1(B)). AMT is different from PMT and its advantage is that it does not required protoplasts for host starter cell types [49]. Some researchers have used protoplasts as starting materials, but in most studies, conidia or germinated conidia are mainly used for starting materials [59,63]. AMT also has several advantages such as high transformation frequency, high gene-replacement frequency, and single-copy T-DNA integration [63]. As a result of conducting several transformation methods such as AMT, PMT, EP, or BT, AMT exerts high transformation efficiency and stability in *A. giganteus* and *A. fumigatus* [51,62]. However, unlike PMT, AMT requires the processes of construction of a binary vector, which containing a fungal selective marker and the gene of interest, and co-culturing *A. tumefaciens* and fungi, so it takes a longer time compared to other transformation methods. To make AMT a success, therefore, it is important to construct the appropriate vectors and optimize the culture condition, such as the ratio of *Agrobacterium*:conidia concentration, cocultivation condition, and acetosyringone concentration, to induce the *vir* gene [63].

Table 2. Lists of AMT in species of *Aspergillus*.

Fungus	Host cell type	Selective markers	*Agrobacterium* strains	Reference
A. awamori	Protoplast	*hph*	LBA1100	[59]
A. niger	Protoplast	*hph*	LBA1100	[59]
A. awamori	Protoplast	*pyrG*	LBA1100	[60]
A. niger	Conidia	*hph*	LBA4404	[61]
A. giganteus	Swollen conidia	*hph*	LBA1100	[51]
A. fumigatus	Conidia	*hph*	EHA105	[62]
A. awamori	Conidia	*hph*	LBA1100	[63]
A. aculeatus	Conidia	*hph*	C58C1	[64]
A. niger	Conidia	*hph*	LBA4404	[65]
A. nidulans	Conidia	*hph*	EHA105	[66]
A. sojae	Conidia	*ble*	LBA4404	[67]
A. terreus	Conidia	*hph*	AGL1	[68]
A. fumigatus	Conidia	*hph*	AGL1	[69]
A. carbonarius	Conidia	*hph*	AGL1	[70]
A. flavus	Conidia	*ble*	AGL1	[71]
A. delacroxii	Conidia	*hph*	AGL1	[72]
A. oryzae	Conidia	*ptrA*	AGL1	[73]
A. luchuensis	Conidia	*hph*	C58C1	[74]
A. niger	Conidia	*hph*	AGL1	[75]

Table 3. Lists of EP-mediated transformation in species of *Aspergillus*.

Strain	Selective markers	Host cell type	DNA type	Reference
A. nidulans UCD1	*argB*	Protoplast	Plasmid (Circular)	[77]
A. nidulans PolArgB	*argB*	Protoplast	Plasmid (Circular)	[77]
A. oryzae ATCC14895	*ben*	Protoplast	Plasmid (Circular)	[78]
A. niger ATCC20739	*argB*	Germinated conidia	Plasmid (Circular)	[79]
A. nidulans RMS011	*argB*	Conidia or germinated conidia	Plasmid (Circular)	[80]
A. fumigatus 237	*hph*	Conidia	Plasmid (Linear)	[81]
A. fumigatus CEA17	*pyrG*	Germinated conidia	Plasmid (Circular)	[82]
A. giganteus IfGB15/0903	*hph*	Conidia	Plasmid (Circular)	[51]
A. fumigatus CEA136	*pyrG*	Germinated conidia	Plasmid (Linear)	[83]
A. fumigatus CEA113	*pyrG*	Germinated conidia	Plasmid (Linear)	[84]

3.3. Electroporation

EP is a highly efficient method for introducing exogenous DNA into a cell by applying a high-voltage electric pulse [76]. EP was used for the first time in *A. nidulans*, and at this time, protoplasts are being used for recipient cells of exogenous DNA [77]. To increase efficiency and reduce time by removing the protoplast preparation process, several research groups used germinated conidia or conidia in *Aspergillus* spp. including *A. oryzae*, *A. niger*, and *A. nidulans* [78–80] (Table 3). In addition, several factors should be optimized, including electric field intensity, pulse condition, DNA concentration, and buffer composition [49]. Although EP is a simple and convenient method compared to other transformation methods, expensive instrumentation is required for EP (Figure 1(C)). Currently, EP is rarely used by researchers for fungal studies.

4. Genetic manipulation tools

Genetic manipulation of filamentous fungi has been critical for understanding fungal biology and developing fungal species for industries. In particular, the identification and production of novel fungal metabolites through genetic manipulation have been used in the pharmaceutical and industrial fields. A variety of genetic manipulation tools have been developed in bacteria or mammalian systems and applied in fungal biology. Of these methods, two genome editing tools, such as homologous recombination (HR)-mediated gene targeting and CRISPR/Cas9 genome editing, are commonly used in fungal research, and they will be discussed below (Figure 2).

4.1. HR-mediated gene targeting

HR-mediated gene targeting is a powerful tool and is most often used for deleting interest of genes, inserting epitope tags, and replacing promoters in species of *Aspergillus* [55]. This method can be used in many investigations on species of *Aspergillus* due to the high transformation efficiency of *ku*-deficient strain, the easy production of PCR-based products, and the optimization of transformation methods and selective markers. Especially, PCR-based cassette construction for HR has the advantage of shortening cloning time and increasing transformation efficiency than cloning by using the vector-based method.

To generate the PCR product for HR, two systems such as the split-marker and fusion (or joint) PCR systems are mainly used [55,85] (Figure 2(A)). The split-marker system requires 2 DNA products containing overlapping fragments of a selective marker such as *pyrG* or *hph (hygB)* [86–88]. Two DNA fragments are introduced into the recipient strains and then these products replace the gene of

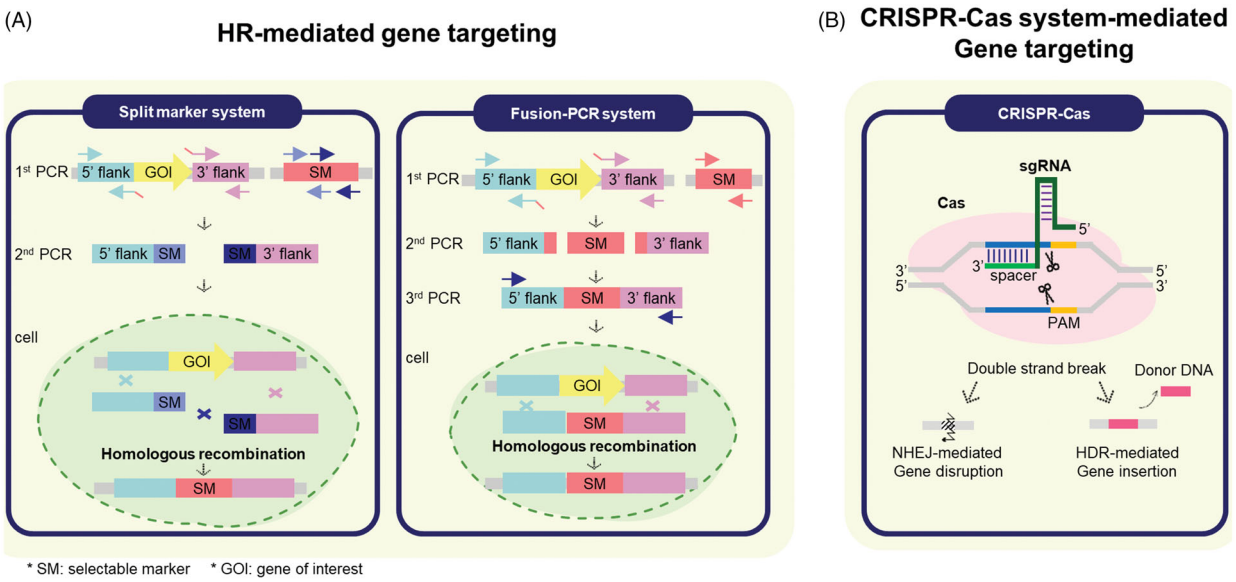

Figure 2. Genome editing methods in species of *Aspergillus*. (A) Procedures of homologous recombination (HR)-mediated gene targeting method and (B) CRISPR-Cas system-mediated gene targeting method used in species of *Aspergillus*.

interest *via* the HR events [89]. The fusion (or joint) PCR system is used more than the split-marker system, and has the advantage of being used for gene tagging and promoter replacement [55,90]. The fusion PCR system generally requires 3 PCR products, 2 fragments for the HR in the genomic DNA flaking a selection region, and the other for a selective marker with or without the tags or the replaceable promoter. These 3 fragments are fused together by joint PCR generating a linear cassette suitable for transformation. The linear PCR cassettes made with the fusion PCR method are generally introduced through PMT methods into the recipient fungal cells, mainly *ku*-deficient strains. This procedure is the most common and widely used method of genetic engineering in species of *Aspergillus*. The use HR-mediated gene targeting is possible only when there are many available selective markers in the fungal species.

4.2. CRISPR–Cas system

In recent years, genome editing using the engineered or bacterial nucleases, such as zinc-finger nucleases, transcription activator-like effector nucleases, and CRISPR–Cas-associated nucleases, have been developed and widely used in almost all eukaryotic systems [91]. Even though these nucleases are also used in filamentous fungi, the CRISPR–Cas system is the most widely used with many filamentous fungi [19]. The CRISPR/Cas system was discovered in the prokaryotic organisms for adaptive immune system against foreign elements and a useful genome editing system has been developed for most eukaryotic systems [92,93]. The CRISPR/Cas system needs 2 components, the Cas9 nuclease and a single chimeric guide RNA (sgRNA) for gene editing in filamentous fungi [19] (Figure 2(B)). The engineered sgRNA, consisting of the Cas-binding site and a spacer that recognizes a protospacer sequence in the specific target site, interacts with the Cas nuclease, which then forms the Cas-sgRNA complex. This complex binds to the target sequence and a short protospacer adjacent motif (PAM), which then make a DNA double-strand break (DSB) within the target DNA (3-5 upstream of the PAM). The DSB in the target site is then repaired by the self-repair mechanism pathways, non-homologous end joining (NHEJ) pathway or homology directed repair (HDR) pathway. The NHEJ pathway is an active repair system but can lead to nucleotide insertions or deletions at DSB site; thereby, it can disrupt the function of the targeted gene. Or, in the presence of donor DNA, the HDR pathway, high-fidelity but less efficient repair system, can insert the donor DNA at the DSB site.

The CRISPR/Cas system was first applied for genome editing system in several species of *Aspergillus* in 2015 [20]. Nodvig et al. generated a vector for the CRISPR/Cas9 system called pFC332 which contains the *hph* gene, the codon-optimized *Streptococcus pyogenes cas9* gene under *tef1* promoter, and the sgRNA genes under the *A. nidulans gpdA* promoter. This vector was introduced through the PMT method into the fungal protoplast and then it disrupted a single target gene in several species of *Aspergillus* (Table 4). Based on this CRISPR/Cas system, several groups are currently studying fungal biology using this system and, in particular, it is used to develop industrially useful strains in *A. niger* and *A. oryzae*. The development and application of the CRISPR/Cas system will be an important

Table 4. Lists of the CRISPR/Cas system in species of *Aspergillus*.

Fugus	CRISPR/Cas system	TM	Purpose	Reference
A. aculeatus *A. brasiliensis* *A. carbonarius* *A. luchuensis* *A. niger*	*A. niger* codon-optimized *Sp*Cas9; *hph* (pFC332), *AnigdpA*(p)-sgRNA-*AnitrpC*(t)	PMT	Disruption of *albA*	[20]
A. nidulans	Codon-optimized *Sp*Cas9; *pyrG*; *AnigdpA*(p)-sgRNA-*AnitrpC*(t)	PMT	Disruption of *yA*	[20]
A. fumigatus	Human codon-optimized *Sp*Cas9; *hph*; *snr52*(p)-sgRNA-*sup4*(t)	PMT	Disruption of *pksP*	[94]
A. fumigatus	Human codon-optimized *Sp*Cas9; *pyr4*; *AfuU6*(p)-sgRNA	PMT	Disruption of *pksP* or *cnaA*	[95]
A. oryzae	Codon-optimized *Sp*Cas9; *niaD*; *U6*(p)-sgRNA-*U6*(t)	PMT	Disruption of *wA, yA, or pyrG*	[96]
A. fumigatus	*In vitro* Cas9 RNPs complex; *hygR* (donor gene)	PMT	Disruption of *pksP*	[97]
A. carbonarius	Codon-optimized *Sp*Cas9; *hph*; *AnigdpA*(p)-sgRNA-*AnitrpC*(t)	PMT	Disruption of *ayg1*	[70]
A. fumigatus	tet^{on} :*Sp*Cas9; *AnigdpA*(p)-spacer-sgRNA-*AnitrpC*(t)	PMT	Single nucleotide insertion in *tynC*	[98]
A. oryzae	*A. oryzae* Codon-optimized *Sp*Cas9; *niaD*; *U6*(p)-sgRNA-*amyB*(t)	PMT	Mutation of *ligD*	[99]
A. nidulans *A. niger* *A. oryzae*	Codon-optimized *Sp*Cas9; *hph*; *AfuU3*(p)-tRNA-sgRNA-*AfuU6*(t)	PMT	Generation of single or multiple mutation	[100]
A. novofumigatus	pFC332, *AnigdpA*(p)-sgRNA-*AnitrpC*(t)	PMT	Disruption of *nvfE*, *nvfK*, or *nvfM*	[101]
A. niger	Codon-optimized *Sp*Cas9; *amdS*; An5SrRNA(p)-sgRNA-T_6	PMT	Disruption of *albA* or *pkaC*	[102]
A. niger	pFC332, donor DNA	PMT	Disruption of *albA*, *glaA*, and *mstC*	[103]
A. kawachii	pFC332; *U6*(p)-sgRNA-*U6*(t):*ptrA*	PMT	Disruption of *amyA*, *brlA*, or *niaD*	[104]

cornerstone for the development of industrial strains and novel fungal metabolites in the future.

5. Conclusions and perspectives

Species of the genus *Aspergillus* have been of great important fungi because of their beneficial and detrimental effects on human. Species of *Aspergillus* can produce various enzymes and organic acids, considering as cell factories. These fungi can produce many secondary metabolites, so it can be utilized as host species for generating novel metabolites that can be used for pharmaceutical purposes. Recent studies reported that species of *Aspergillus* can decompose plastics and pollutants, so these fungi can be utilized for the environmental field. Furthermore, through the development of genetic engineering and systemic biology, many researchers sought to use filamentous fungi as cell factories. Unlike bacteria or yeast, the available gene editing methods and transformation efficiency are low, but filamentous fungi are still attractive cell factories for industry. To develop *Aspergillus* as cell factories, currently, PMT and HR-mediated gene targeting are mainly used in genetic engineering. Recently, the CRISPR genome editing method was used for gene editing, but the use of other gene editing methods developed in bacteria and yeast is still marginal. Therefore, it is necessary to develop a more efficient transformation method and a system for genetic manipulation.

Disclosure statement

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