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Strategy for efficient cloning of biosynthetic gene clusters from fungi

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Filamentous fungi are excellent sources for the production of a group of bioactive small molecules which are often called secondary metabolites (SMs). The advanced genome sequencing technology combined with bioinformatics analysis reveals a large number of unexplored biosynthetic gene clusters (BGCs) in the fungal genomes. To unlock this fungal SM treasure, many approaches including heterologous expression are being developed and efficient cloning of the BGCs is a crucial step to do this. Here, we present an efficient strategy for the direct cloning of fungal BGCs. This strategy consisted of Splicing by Overlapping Extension (SOE)-PCR and yeast assembly *in vivo*. By testing 14 BGCs DNA fragments ranging from 7 kb to 52 kb, the average positive rate was over 80%. The maximal insertion size for fungal BGC assembly was 52 kb. Those constructs could be used conveniently for the heterologous expression leading to the discovery of novel natural products. Thus, our results provide an efficient and quick method for the low cost direct cloning of fungal BGCs.

biosynthetic gene clusters, *Saccharomyces cerevisiae*, homologous recombination, DNA assembly

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INTRODUCTION

Fungal secondary metabolites (SMs) are important sources for the development of modern drugs, and the application of penicillin reveals the era of fungal SMs mining and drug discovery (Clutterbuck et al., 1932). It is estimated that there are between 500,000 and 3 million ascomycete fungal species on earth (Blackwell, 2011), and each fungus contains 50 to 90 secondary metabolite biosynthetic gene clusters (BGCs) (Khaldi et al., 2010; Blackwell, 2011; Inglis et al., 2013; Han et al., 2016). However, a large number of BGCs are cryptic under laboratory growth conditions and require genetic manipulation to activate them (Brakhage and Schroeckh, 2011). From the Aspergillus Genome Database (AspGD; http://www.aspgd.org/), only about 30.4% of BGCs were identified in the model fungal *Aspergillus nidulans*, leaving nearly 70% waiting to be excavated.

To take full advantage of the tremendous resource provided by fungi, there are several strategies being proposed to induce the expression of cryptic secondary metabolite BGCs, including changing the culture condition, interaction with other microbes, epigenetic regulation, genetic manipulation of the global-like regulator and heterologous expression (Wiemann and Keller, 2014; Fan et al., 2017; Niu et al., 2017; Zheng et al., 2017a; Zheng et al., 2017b; Zhuo et al., 2017; Lin et al., 2018; Zhou et al., 2019). Among these, expression of BGCs in heterologous hosts offers a more effective strategy to awaken the cryptic BGCs, which can

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complete the direct studies of SMs in wild type fungi (Reen et al., 2015). Using heterologous expression, a cryptic BGC en-coding neosartoricin B from dermatophytes had been successfully expressed in *A. nidulans* (Yin et al., 2013). Besides, the heterologous expression strategy could be a better option for studying pathways of cryptic compound biosynthesis and improving the yield of natural products (Ma et al., 2018).

The basis of heterologous expression is the recombinant construction of large DNA fragments of BGCs. However, it is impossible to complete large DNA fragment assembly by using the classic method of restriction-ligation because it is time-consuming and has low-efficiency (Cohen et al., 1973). With the advent of modern molecular biotechnology, several methods have been developed, such as Gibson assembly, transformation-associated recombination (TAR) cloning and yeast assembly by SOE-PCR (Ma et al., 1987; Larionov et al., 1996; Gibson et al., 2009). Although the Gibson assembly with an exonuclease-based mechanism is more time-saving and has a high success rate, it costs too much and hardly constructs a plasmid containing large inserted fragments which cover the sizes of fungal BGCs, which range from 30 kb to 100 kb (Clevenger et al., 2017). TAR cloning is used for isolating the selective chromosomal segments from complex genomes by yeast homologous recombination *in vivo* (Larionov et al., 1996). However, the large DNA fragments hooked by overlap region on the vector were usually larger than the targeted BGCs, it is hard for us to select the appropriate digestion position in genomic DNA. What's more, the efficiency of the TAR strategy is still low, with the yield of gene-positive clones varying from 1% to 5% (Kouprina and Larionov, 2008). No successful case is available of using TAR in filamentous fungi.

Over all, yeast assembly is a more suitable choice in the field of larger DNA fragment assembly for construction of fungal BGCs heterologous expression vector, because this strategy can not only handle the assembly of large DNA segments, but also possesses high efficiency and accuracy (Muller et al., 2012). The mechanism of yeast assembly was based on splicing by overlapping extension (SOE)-PCR in which the large gene cluster can be divided into small DNA fragments and experienced homologous recombination in the yeast strain *Saccharomyces cerevisiae* (Ma et al., 1987; Shinohara and Ogawa, 1995; Paques and Haber, 1999; Kupiec, 2000). In this report, this method is comprehensively presented for the application of fungal BGC construction. More important, we have tested the recombinant rate of different length of BGCs or genes which ranging from 7 kb to 52 kb. Based on this test, we expect that this method will be further advanced and applied for construction of large DNA fragments.

RESULTS

Construction of fungal BGCs up to 52 kb by yeast assembly

Bioinformatics analysis of the known fungal genomes reveals that 90% of gene clusters of the secondary metabolites are "silent" (Brakhage and Schroeckh, 2011). The use of heterologous expression to activate fungal "silent" gene clusters is an effective current approach to discover new natural products and their biosynthetic pathways (Bilyk et al., 2016). Here, we tested the recombinant rate of 14 different lengths of BGCs or core genes which range are from 7 kb to 52 kb. The sizes were evenly distributed between the classes of less than 20 kb, 20 kb to 30 kb, and larger than 40 kb (Figure 1). Moreover, there were several principles for selecting BGCs or genes for future study. For example, we had selected the BGCs containing NRPS-PKS hybrid synthetase, because this hybrid possessed special structures and was relatively rare in fungi kingdom, suggesting that they could produce novel structure compounds. Meanwhile, we chose the BGCs clusters containing transcription factors (TFs). If the heterologous expressing transformants didn't produce new compounds or produced only a few new compounds, we could regulate the expression of the TFs or the core genes to improve the production of these compounds in the heterologous host *A. nidulans* (Zhang et al., 2017).

A schematic diagram illustrating the overview of our strategy is summarized in Figure 1 to facilitate understanding of the process. The unit fragments were obtained by using the SOE-PCR strategy, and then the fragments with overlap and linearized vector were co-transformed into yeast competent cells for homologous recombination. The positive yeast colonies were verified by PCR using designated primers, and the recombinant plasmids were extracted from the positive yeast colonies and transformed into *E. coli* for propagating and recombinant plasmid verification by restriction enzyme digestion. Although the whole process takes about 5 days, several recombinant plasmids can be obtained simultaneously.

The efficiency statistics of yeast assembly on different sizes of inserted fragments

Here, we explored the efficiency of yeast assembly on different sizes of DNA fragments (Figure 2C, Table 1). We divided them into three size classes according to the BGCs or genes sizes, including less than 20 kb, 20 kb to 30 kb and larger than 40 kb. In the group of less than 20 kb, we have constructed 5 plasmids in which the inserts were 7, 8, 10, 12 and 16 kb length, respectively. Colony PCR showed that their yeast colonies positive percentage were 36.4%, 92.7%, 100%, 76.1% and 81.8%, and *E. coli* colonies positive percentage were 100%, 100%, 75%, 66.7% and 100%, respec-

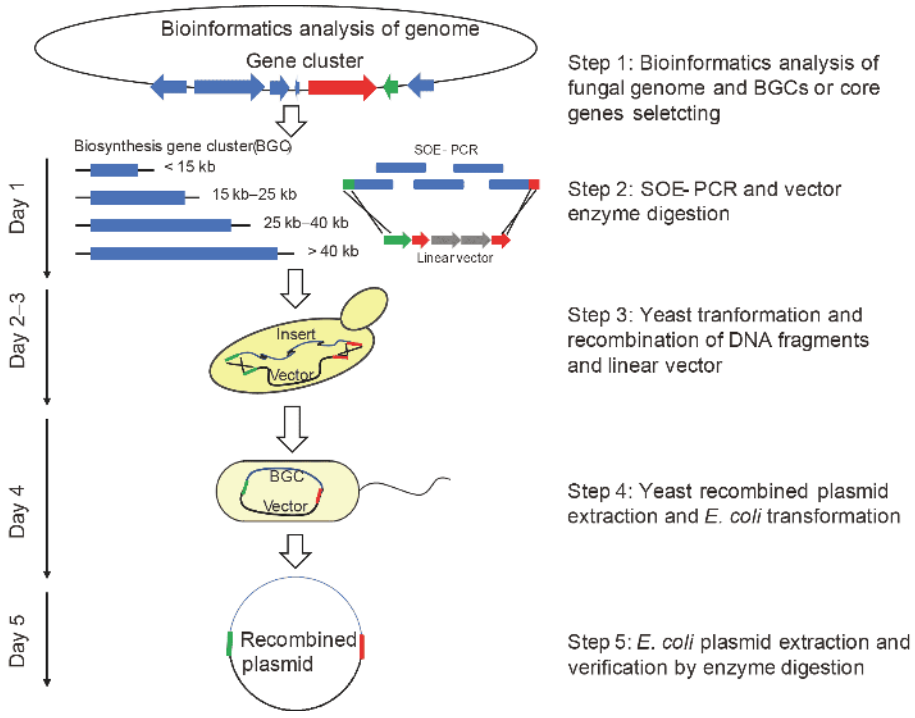

Figure 1 (Color online) Construction of fungal BGCs or genes by yeast assembly method. This method is based on SOE-PCR and yeast homologous recombination. Bioinformatic analysis was utilized to study the fungal genome and select BGCs or core genes. Targeted BGCs or core genes were divided into several DNA fragments and they were amplified by PCR from fungal genomic DNA, respectively. After that, targeted DNA fragments and linearized vector were co-transformed into yeast cells, then DNA fragments would assemble into the linearized vector by homologous recombination. Following this yeast transformants were screened by diagnostic PCR, then the recombinant plasmid was isolated from positive yeast colonies. Finally, the recombinant plasmid was enriched in *E. coli* and digested by restriction enzymes for verification. A recombinant plasmid would be constructed within 5 days by this method.

tively (Table 1). In the group of size of inserts ranged from 20 kb to 30 kb, we have constructed 5 plasmids in which the inserts were 23, 27, 27, 28 and 30 kb length, respectively. Their yeast colonies positive percentage were 36.4%, 100%, 68.2%, 50% and 75%, and *E. coli* colonies positive percentage were 67%, 100%, 70%, 80% and 100%, respectively (Table 1). In the group of size of inserts was larger than 40 kb, we have also constructed 4 plasmids in which the inserts were 40, 47, 48 and 52 kb length, respectively. During the process of plasmid assembly, their yeast colonies positive percentage were 76.1%, 56.3%, 66.7%, and 3%, and *E. coli* colonies positive percentage were 75%, 100%, 100% and 100%, respectively (Table 1).

By analyzing the data of these three groups, average yeast positive percentage of <20 kb, 20−30 kb, >40kb were 77.4%, 65.9% and 50.1%, respectively (Figure 2A). This result suggested that the bigger the inserted fragments are, the more difficult to assemble them in yeast. As for *E. coli* colonies, the average positive percentage of these three groups were 88.3%, 88.3% and 93.8%, respectively (Figure 2B). The positive percentage was independent of the size of inserts and stabilized at over 80% in *E. coli* colonies (Figure 2B).

These positive recombinant plasmids were identified by enzymatic digestion, and we present here the 12, 27, 40 and 48 kb BGCs as examples (Figure 2C). The gel electrophoresis showed that the bands of enzyme digestion were identical with the predicted results by the Snapgene software (Figure 2C and D). From the above analysis we thought that the important process for the large BGCs construction was to improve the positive rate in yeast assembly stage. We sequenced 8 positive recombinant plasmids, with inserts of 7, 8, 10, 12, 16, 28, 30 and 48 kb. Mutation rates were calculated after alignment, there is only one mismatch was found in the 48 kb insertion. The others were all correct (Table 2). Thus, we believe our strategy is an efficient, low cost and quick method for the direct cloning of fungal BGCs.

DISCUSSION

In this study, we developed and evaluated an effective method to obtain the fungal cryptic biosynthetic gene clusters, called splicing by overlapping extension (SOE)-PCR homologous recombination in yeast. We tested the positive rate of yeast assembly of different length BGCs ranging from 7 to 52 kb both in yeast and bacteria, indicating that the key step for construction of the large BGCs is to improve the positive success rate in the yeast assembly stage.

Since more and more fungal genomes have been sequenced, it has been found that fungi possess a lot of sec-

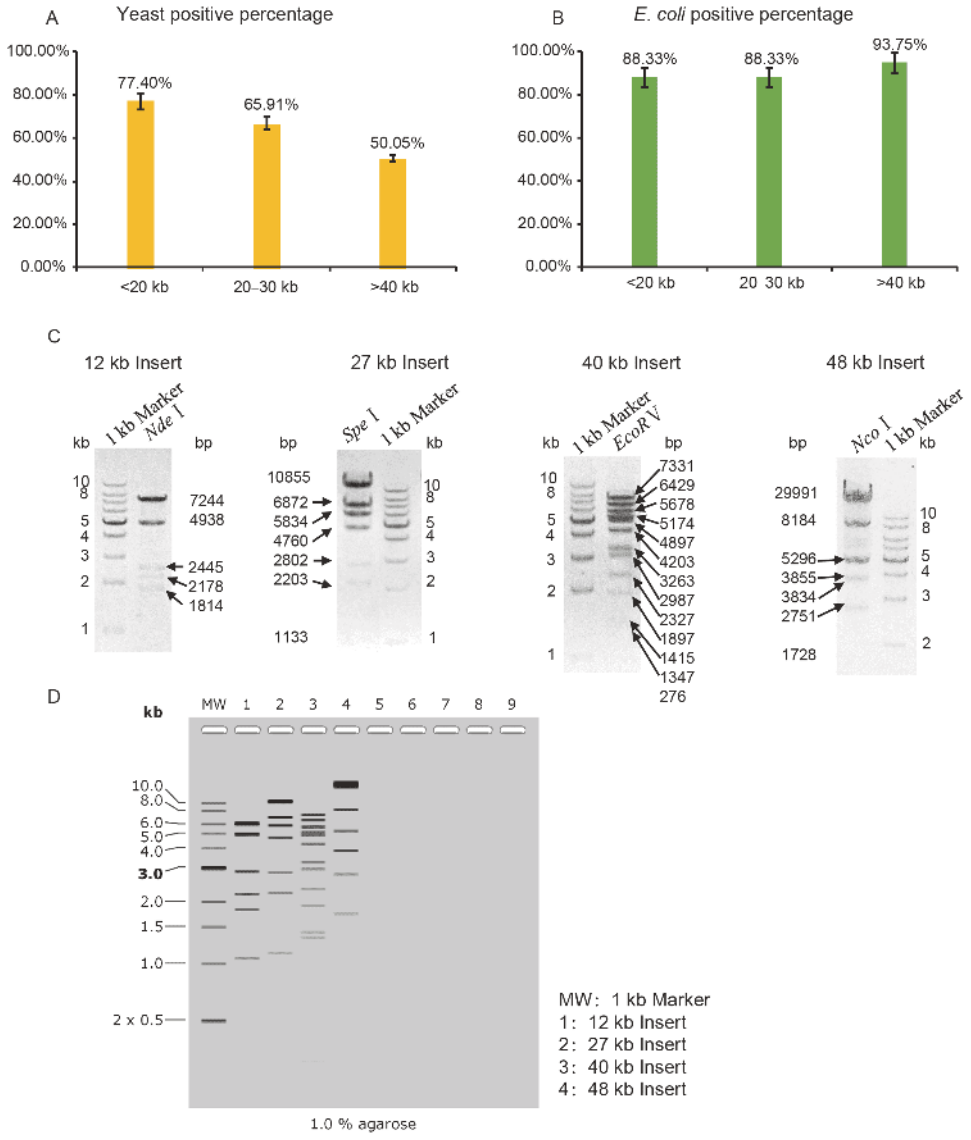

Figure 2 (Color online) The average positive percentage of yeast and *E. coli* colonies and positive plasmids determination by enzyme digestion. A, In accordance with the size of inserts, these data were clustered into three groups. The average positive percentage showed a decrease with the increase of the insert length in the yeast. B, The average positive percentage stabilized at over 80% in the *E. coli* colonies PCR. C, We used these four typical positive recombinant plasmids as examples. These plasmids were digested by *Nde* I, *Spe* I, *EcoR* V and *Nco* I, respectively. The digested bands agreed with predicted result by the Snapgene software. D, The four typical positive recombinant plasmids were digested by *Nde* I, *Spe* I, *EcoR* V and *Nco* I, respectively. The digested bands were predicted by Snapgene software.

ondary metabolites BGCs, indicating that fungi have a tremendous potential to supply natural products which has attracted enormous attention for drug discovery. Heterologous expression is an effective strategy to express or activate BGCs, but construction of large fungal gene clusters is still challenging. Here, we showed the advantage of the yeast assembly which had less requirements on genome quality, and the operation was more flexible and simplified than the TAR strategy. Moreover, TAR needs comparatively intact genomic DNA to ensure the integrity of the digested BGCs. So far, using the approach of yeast assembly, we have successfully constructed many uncharacterized fungal BGCs, PKSs and NRPSs in our lab. And some of them have been expressed in the heterologous host *A. nidulans* (Zhang et al., 2017; Li et al., 2018; Ma et al., 2018).

In addition to the fungal BGCs, the yeast assembly can also be applied in various constructions including removing introns, tandem co-expression of several genes which are distributed in different positions in the genome and so on. For example, if one gene possesses many introns in its genome sequence and exists in a silent state in normal culturing condition, it will be difficult to amplify from a cDNA library. The cost of synthesis is very expensive. Therefore, the ORF region can be effectively obtained through yeast

Table 1 The statistics of efficiency of yeast assembly method

Inserts Size/kb		Yeast positive colonies	Yeast total colonies	Positive percentage	*E. coli* positive colonies	*E. coli* total colonies	Positive percentage
<20	7	8	22	36.4%	8	8	100.0%
	8	51	55	92.7%	22	22	100.0%
	10	20	20	100.0%	6	8	75.0%
	12	35	46	76.1%	8	12	66.7%
	16	18	22	81.8%	8	8	100.0%
20–30	23	20	55	36.4%	4	6	66.7%
	27	22	22	100.0%	3	3	100.0%
	27	15	22	68.2%	7	10	70.0%
	28	7	14	50.0%	8	10	80.0%
	30	6	8	75.0%	1	1	100.0%
≥40	40	35	46	76.1%	6	8	75.0%
	47	36	64	56.6%	6	6	100.0%
	48	4	6	66.7%	16	16	100.0%
	52	2	67	3.0%	20	20	100.0%

Table 2 Mutation rates in sequenced insertions of biosynthetic gene clusters

Insert size	Gap mutation	Insert mutation	Mismatch mutation	Mutation rates
7 kb	0	0	0	0
8 kb	0	0	0	0
10 kb	0	0	0	0
12 kb	0	0	0	0
16 kb	0	0	0	0
28 kb	0	0	0	0
30 kb	0	0	0	0
48 kb	0	0	1	0.000208%

assembly technology. Firstly, exons are amplified from genomic DNA with designated primers which contain homologous sequences with the lateral side of upstream or downstream exons or with the linearized vector. After transforming these exons, fragments and linearized vector into yeast, we can obtain the vectors with only ORF regions of targeted genes for the further experiments. In the same way, we can amplify some non-adjacent genes from genomic DNA with primers containing the homologous sequence with the lateral side of upstream or downstream genes or with the linearized vector, and these genes will be constructed in one vector by homologous recombination in yeast.

From the comparison on the positive rate of different sizes of assembled fragments, it is suggested that the positive rate of *E. coli* colonies remains at a higher level (over 80%), regardless of the size of inserted fragments, but in yeast, the larger the inserted fragments were, the more difficult it was to assemble them. However, another reason for the low positive rate was that the linearized vector induced self-ligation when it was transformed into yeast. It led us to select a large number of negative yeast colonies with only vector when we performed the colony PCR, resulting in a low positive rate of yeast colonies. If we can increase the positive rate of yeast colony PCR, it could save time and cost.

Blue-white screening has been widely used in the screening of positive *E. coli* colonies. The *E. coli* strain with recombinant plasmid were cultured on screening medium containing X-gal. After growth overnight, the colonies would show blue or white color, indicating the carriage of the vector alone or vector with inserted DNA, respectively (Chaffin and Rubens, 1998). If there is a simpler and more effective screening mechanism that can be introduced into the yeast transformants screening system, the positive rate of yeast transformation could be increased.

MATERIALS AND METHODS

Plasmid, strains and media

p*YH-wA-pyrG*, as an *E. coli*-yeast-*Aspergillus* shuttle vector, was used for assembly with the divided fragments of BGCs in yeast in this study. It consists of a yeast centro-mere se-

quence (CEN), and an autonomously replicating sequence (ARS) (Mayorga and Timberlake, 1990; Fu et al., 2012). It contains an ampicillin resistant gene for *E. coli* transformants screening, and an URA3 coding gene for yeast transformants selecting by plating on medium without uracil. In addition, it possessed an *Aspergillus fumigatus pyrG* (*AfpyrG*) coding gene for screening *Aspergillus* transformants in the medium without uracil and uridine. The auxotroph yeast strains used in this study was *Saccharomyces cerevisiae* strain BJ5464-NpgA (MATα *ura*3-52 *his*3-Δ200 *leu*2-Δ1 *trp1pep4*::HIS3 *prb1*Δ1.6R *can1* GAL). Yeast transformants were selected and inoculated in synthetic dextrose complete medium (SDCt) with appropriate supplements corresponding to the auxotrophic markers (Wu et al., 2016). *E. coli* strains DH5α (ThermoFisher SCIENTIFIC, China) and EPI300 (TransforMaxTM, USA) were used as the host for plasmids propagation, and they were cultured in LB medium with appropriate antibiotics. Compared to the DH5α strain, the EPI300 strain can contain larger plasmids, so that it is much more suitable for fungal BGCs reproduction.

Fungal genomic DNA isolation and standard molecular biology techniques

For the genomic DNA acquisition, *Pestalotiopsis fici* CGMCC3.15140, *Aspergillus fumigatus* AF293 and *Glarea lozoyensis* ATCC20868 were cultured. *P. fici* was grown on grown at 25°C in Potato Dextrose Broth (PDB) and *A. fumigatus* was grown on liquid minimal medium (LMM) (Shimizu and Keller, 2001; Zhang et al., 2003; Fekete et al., 2012; Xu et al., 2014). Also, the strain of *G. lozoyensis* ATCC 20868 was obtained from American Type Culture Collection (ATCC) and cultured on LYCP-5 medium described by Connors et al. (Connors et al., 2000). The genomic DNA isolation modifications involved the following steps: (1) Mycelia were harvested by filtration over nylon mesh and thoroughly washed with sterile distilled water. Excess liquid was removed by squeezing between paper sheets. Then, the dried tissue was added into a 1.5 mL tube; (2) 700 μL LETS buffer (0.1 mol L^{-1} LiCl, 20 mmol L^{-1} EDTA, 10 mmol L^{-1} Tris-HCl, and 0.5% SDS) was added into the tube, and homogenized with the tissues by grinding; (3) 700 μL Solarbio® DNA extraction reagent PCI (Phenol: Chloroform: Isoamylol=25:24:1) was added and mixed thoroughly; (4) the mixture was incubated at room temperature for about 10 minutes, then centrifuged at 13,000 r min^{-1} for 10 minutes at 4°C. The clean supernatant was transferred into a new tube; (5) 1 mL 95% ethanol (v/v) was added into the supernatant and mixed thoroughly for DNA precipitation; (6) the mixture was centrifuged at 13,000 r min^{-1} for 10 minutes at 4°C and the supernatant was discarded; (7) 300 μL 75% ethanol (v/v) was used to wash the DNA pellet attached to bottom of the tube; (8) the DNA pellet was dried and resus-pended it by ddH_2O with RNase A (OMEGA bio-tek, USA) to eliminate any RNA; (9) then the DNA solution was heated for 30 minutes in 65°C to inactivate RNase A.

PCR reactions were carried out using either Q5 high-fidelity DNA polymerase (New England Biolabs, USA) or TransStart® *FastPfu* DNA polymerase (Transgene Biotech, China) on a T100TM Thermal cycler from Bio-Rad. In order to screen transformants, the diagnostic PCR were performed using 2×Taq Mix kit (TIANGEN BIOTECH, China). All the restriction enzymes used in this study were purchased from New England Biolabs. All the enzymes were used following the recommendation of the manufacturers.

Preparation of targeted DNA fragments and linearized vector

The basic strategy of this method was SOE-PCR and yeast homologous recombination. The general principle and operating protocol are presented in Figure 1. Here, we introduced the detailed process of constructing an *AfuPKS* cluster as an example for explaining this method. Firstly, the 8 DNA fragments with overlap region were amplified from *A. fumigatus* genomic DNA with the designed primers (Table 3).

Two of them were about 3 kb, and others were about 5 kb (Figure 3A). The overlap region was about 150 bp between two adjacent DNA fragments, and two ends of DNA fragments contained 39 bp overlaping sequences with flanking of the NheI cutting site of p*YH-wA-PyrG* (Figure 3A). PCR reaction for amplifying the *AfuPKS* gene cluster was carried out using TransStart® *FastPfu* DNA polymerase (Transgene Biotech) (Table S1). The p*YH-wA-PyrG* was linearized by using restriction enzymes NheI. These fragments and the linearized vector were purified with the Quick Gel Extraction Kit (Transgene Biotech). Thehe concentration of purified vector and DNA fragments was quantified by NanoDrop or agarose gel electrophoresis (Figure 3B and C).

Yeast transformation and positive yeast colonies screening

Firstly, the high-quality yeast competent cells were required which was the key factor for yeast assembly success. In this study, the yeast competent cells were made by using *S.c.* EasyComp Transformation Kit (Invitrogen, USA) according to manufacturer's instructions. During the process of yeast transformation, the equimolar DNA segments and linearized vector were added into yeast competent cells, and the quantity of vector that was usually used was about 250 ng. According to the concentration analysis of DNA fragments and linearized vector (Figure 3B and C), the amounts for yeast transformation are presented in Table S2. DNA frag-

Table 3 Primers for *AfuPKS* amplification used in this study

Primers	Oligonucleotide sequence (5'−3')	Uses
Fragment_1_F	TTGCCTGATCAGCGGACTTGACTCTCCTTCTCCTGATCGC TTGTCTTGGGGTGGTTGCC	Amplification of cluster fragment 1
Fragment_1_R	CGTGCGATCATTGATGAACTGG	
Fragment_2_F	GAACAACTTCACGACCATTGCG	Amplification of cluster fragment 2
Fragment_2_R	CTGAAGTGCACCAGCTCGTC	
Fragment_3_F	CCTTGCCGCAGGTATTCAGG	Amplification of cluster fragment 3
Fragment_3_R	CTATGTTGTGGCGACGACCC	
Fragment_4_F	GCCTAGACAGTATCCTCCCCG	Amplification of cluster fragment 4
Fragment_4_R	GAGGAACAATAGGCGCGTCG	
Fragment_5_F	CACCAAACCACGGAACCAAC	Amplification of cluster fragment 5
Fragment_5_R	CCATGGCTTGATGGATTCAGCC	
Fragment_6_F	GACGGACTGTAAGAGAGCCG	Amplification of cluster fragment 6
Fragment_6_R	CTGTGTTCGAAAGCCCTCAG	
Fragment_7_F	CCGTTATCTCCAACACGCTCC	Amplification of cluster fragment 7
Fragment_7_R	CGGTCTTTTTCTTAGCCGACG	
Fragment_8_F	GCCTGGTCGATCTTGTAGGC	Amplification of cluster fragment 8
Fragment_8_R	CGGCGGATATCCATGGAACCCTAGCGAGAGTTATTCTGT GTCTGACGAAATATGTTGTG	

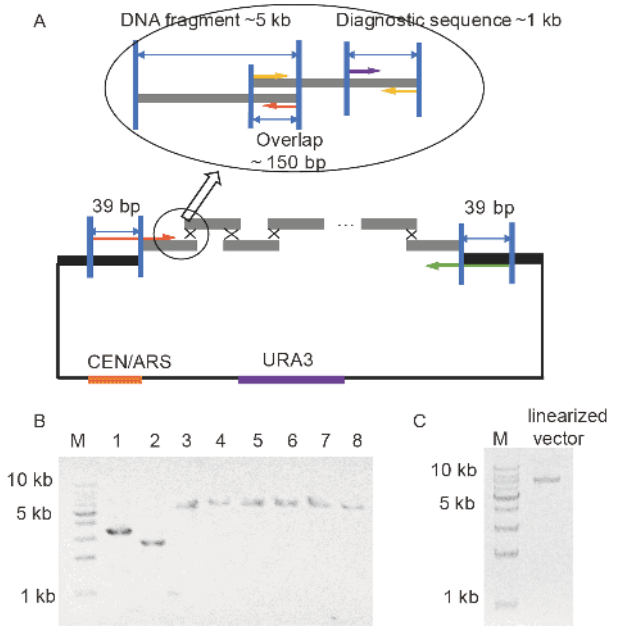

Figure 3 (Color online) Schematic diagram for large fungal DNA fragment assembly. A, The strategy was based on the SOE-PCR. The first forward primer (Fragment_1_F, shown by red arrow) and the last reverse primer (Fragment_8_R, shown by green arrow) had a 39 bp overlap with the linearized vector. The two adjacent DNA fragments shared about 150 bp overlapping region for homologous recombination in yeast. 1 kb diagnostic sequence was used for colony PCR (shown by violet and yellow arrow). B, Agarose gel electrophoresis analysis of purified DNA fragments. Lane M was 1 kb marker, and lines 1–8 represented the consecutive DNA fragments, respectively. The concentration of these fragments were about 400 ng μL^{-1}, 300 ng μL^{-1}, 100 ng μL^{-1}, 100 ng μL^{-1}, 150 ng μL^{-1}, 150 ng μL^{-1}, 150 ng μL^{-1} and 100 ng μL^{-1}, respectively (note: the marker was loaded about 6 μL and the samples was loaded about 0.5 μL). C, The concentration of the linearized vector was about 160 ng μL^{-1} (note: the marker was loaded about 6 μL and the sample was loaded about 0.5 μL).

ments, linearized vector, and 500 μL Solution III were added into 50 μL yeast competent cells, and the mixture was vortexed for 1 second. Afterwards, the mixture was incubated at 30°C for 1 hour, and vortexed for 1 second every 15 minutes. The homogenized mixture was inoculated on selective medium SDCt and incubated at 30°C. After 2 days, the yeast colonies could be visible. We selected about 30−100 monoclonal colonies and inoculated them on a new selective medium SDCt by using the toothpicks. After 24 hours growth of monoclonal yeast, colony PCR was used to analyze whether they were positive or not (Table S3). Then, the "positive" colonies were inoculated on another new SDCt plate, and they were verified by colony PCR (Table S3).

Finally, the yeast plasmids were isolated from the positive colonies by ZymopreTM Yeast Plasmid Miniprep kit (Zymo Research, USA). In this process, it is worth noting that the volume of yeast cell should be under 50 μL. Otherwise, the cell wall couldn't be degraded thoroughly by Zymolyase. Moreover, the plasmids from yeast cells should be firstly detected by PCR, and then transformed into DH5α or EPI300. After the bacterium matured, the colony PCR was performed for screening positive transformants. The transformants were then incubated overnight in liquid LB at 37°C and 200 r min^{-1}, and the plasmids prepared using the Plasmid Mini kit I (OMEGA bio-tek).

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

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SUPPORTING INFORMATION

Table S1 SOE-PCR reaction system for obtaining the overlapping fragments

Table S2 Yeast transformation protocol

Table S3 Yeast colony PCR reaction

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