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Rapid Assembly of Yeast Expression Cassettes for Phenylpropanoid Biosynthesis in *Saccharomyces cerevisiae*

(Pemasangan Pantas Gen Kaset Yis untuk Penghasilan Fenilpropanoid dalam *Saccharomyces cerevisiae*)

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ABSTRACT

Microbial production of natural products using metabolic engineering and synthetic biology approaches often involves the assembly of multiple gene fragments including regulatory elements, especially when using eukaryotes as hosts. Traditional cloning strategy using restriction enzyme digestion and ligation are laborious and inflexible owing to the high number of sequential cloning steps, limited cutting sites and generation of undesired 'scar' sequences. In this study, a homology-based isothermal DNA assembly method was carried out for one-step simultaneous assembly of multiple DNA fragments to engineer plant phenylpropanoid biosynthesis in Saccharomyces cerevisiae. *Rapid construction of yeast plasmid harboring dual gene expression cassettes was achieved via isothermal assembly of four DNA fragments designed with 20 bp overlapping sequences. The rate-limiting enzyme of phenylpropanoid pathway, cinnamate 4-hydroxylase encoded by* C4H *gene from* Polygonum minus *was cloned in tandem with yeast promoter and terminator elements of* S. cerevisiae *for efficient construction of phenylpropanoid biosynthetic pathway in recombinant yeast. The assembled pAG-CAT (C4H-ADH1t-TEF1p) shuttle plasmid and transformation of* S. cerevisiae *with the plant* C4H *gene were confirmed via PCR analysis. Based on these findings, the yeast shuttle plasmid harboring* P. minus *phenylpropanoid biosynthesis gene was efficiently constructed to be the starting platform for the production of plant natural products in genetically-engineered* S. cerevisiae.

Keywords: Phenylpropanoid biosynthesis; Polygonum minus*; rapid DNA assembly;* Saccharomyces cerevisiae*; synthetic biology*

ABSTRAK

Penghasilan produk semula jadi oleh mikrob melalui kaedah kejuruteraan metabolik dan biologi sintetik sering melibatkan pemasangan serpihan gen berganda termasuk elemen gen pengawalselia yang penting untuk sistem eukariot. Pengklonan gen secara tradisi menggunakan enzim pemotongan dan penyambungan DNA adalah sukar dan tidak fleksibel kerana bergantung kepada langkah pengklonan berjujukan yang sangat banyak, ketidaksesuaian tapak pemotongan dan penghasilan jujukan 'parut' yang tidak dikehendaki. Kajian ini melaporkan pemasangan berbilang fragmen DNA berganda secara serentak dan dalam satu langkah melalui kaedah pemasangan DNA isoterma untuk penghasilan fenilpropanoid dalam yis Saccharomyces cerevisiae. *Pembinaan plasmid konstruk yis telah berjaya dilakukan dengan pantas melalui kaedah pemasangan isoterma empat fragmen DNA yang telah direka untuk mengandungi jujukan bertindih sebanyak 20 pasangan bes. Enzim sinamat 4-hidrolase (C4H) daripada* Polygonum minus *yang merupakan enzim pengehad kadar fenilpropanoid, telah dipasang bersama elemen penggalak dan penamat yis untuk pembinaan laluan fenilpropanoid dalam* S. cerevisiae *rekombinan secara cekap dan pantas. Hasil pemasangan plasmid lengkap pAG-CAT (C4H-ADH1t-TEF1p) dan transformasi gen* C4H *dalam* S. cerevisiae *telah disahkan melalui analisis tindak balas rantai polimerase (PCR). Berdasarkan hasil kajian ini, plasmid ulang-alik yis yang mengandungi gen biosintetik fenilpropanoid daripada* P. minus *telah berjaya dibina dengan cekap dan akan dijadikan sebagai landasan pemula untuk penghasilan produk semula jadi menggunakan* S. cerevisiae *yang terubah suai secara genetik.*

Kata kunci: Biologi sintetik; biosintesis fenilpropanoid; pemasangan DNA pantas; Polygonum minus*;* Saccharomyces cerevisiae

INTRODUCTION

Advances in metabolic engineering and synthetic biology fields have rapidly enabled the production of pharmaceuticals, natural products and biofuels using microorganisms as cell factories (Smanski et al. 2016; Stephanopoulos 2012). *Escherichia coli* and *Saccharomyces cerevisiae* are two of the most established microbial systems for the production of various products (Liu et al. 2013). Production of natural products such as terpenoids, flavonoids and alkaloids often requires the construction of multiple gene fragments for the expression of corresponding biosynthetic pathways

(Galanie et al. 2015; Trantas et al. 2009). Molecular cloning of genes from various genetic sources have been well established using the restriction enzyme digestion and ligation methods of the gene of interests in bacterial plasmid vectors (Cohen et al. 1973; Sambrook et al. 1989). Although effective, this cloning method is unsuitable for high throughput and rapid DNA assembly of multiple fragments due to rigid requirements of restriction enzyme sites and lack of versatility in vector design (Festa et al. 2013; Hartley et al. 2000).

The restriction enzyme-based techniques are limiting multiple gene cloning and seamless eukaryotic plasmid construction due to the requirements of promoter and terminator regulatory regions for driving and regulating the expression of the individual coding sequences (Blount et al. 2012; Hawkins & Smolke 2008). A standardized method that enables rapid assembly of multiple gene fragments for metabolic engineering and synthetic biology applications would be particularly useful in rapid eukaryotic strain development especially for the biosynthesis of plant metabolites that often requires the construction of complex biosynthetic pathways.

For this purpose, this study was designed to demonstrate the application of the homology-based DNA assembly technique for rapid and efficient construction of yeast plasmid to express plant phenylpropanoid biosynthesis gene in recombinant *S. cerevisiae*. Here we report the assembly of cinnamate 4-hydroxylase (*C4H*) gene from medicinal plant *Polygonum minus* with yeast transcriptional regulatory elements using isothermal DNA assembly. A specially-designed strategy was devised for rapid construction of double expression cassettes that will enable co-expression of genes for the plant phenylpropanoid biosynthesis pathway in recombinant yeast.

Materials and Methods

Strains and Cultivation Conditions

For plasmid propagation and molecular cloning, *E. coli* TOP10 strain was used. Luria Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract and 10 g/L sodium chloride) with 100 µg/mL ampicillin or 50 µg/mL kanamycin was used for plasmid preparation and maintenance. *S. cerevisiae* CEN.PK 2-1C was used for yeast transformation and plasmid propagation. YPD medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L dextrose) was used for yeast cultivation and synthetic dropout (SD) medium lacking histidine (SD-His^-) was used for yeast transformant selection (Sherman 1991). Bacterial and yeast cells were grown at 37°C and 30°C, respectively, with shaking at 200 rpm where appropriate. *P. minus* was grown in an experimental plot at Universiti Kebangsaan Malaysia, Bangi. Fresh leaves of *P. minus* were sampled for the RNA isolation and cDNA synthesis (Loke et al. 2017).

Nucleic Acid Isolation, Amplification and Sequencing

Isolation of total RNA and cDNA synthesis from *P. minus* leaf was respectively carried out using Spectrum™ Plant Total RNA Kit (Sigma Aldrich, USA) and Maxima First Strand cDNA Synthesis Kit (Thermo Scientific, USA) according to the manufacturer's instructions. Plasmid isolation in bacterial and yeast cells was performed using Zyppy™ Plasmid Miniprep Kit and Zymoprep™ Yeast Plasmid Miniprep II (Zymo Research, USA), respectively, by following the kit instructions. PCR products were purified using NucleoSpin® Gel and PCR Clean-up Kit (Macherey-Nagel, USA). DNA primers synthesis and sequencing analysis were performed by First BASE Laboratories (First BASE Laboratories, Malaysia). High fidelity DNA polymerases, Q5 DNA polymerase (New England Biolabs, USA) and KOD FX Neo (Toyobo, Japan) were used for high fidelity amplification of DNA fragments. DpnI restriction enzyme (New England Biolabs, USA) was used to treat vector fragment to reduce vector background in *E. coli* transformants.

Construction of Yeast Plasmid

Yeast plasmid pAG423GPD-ccdB-Cerulean (Addgene, Cambridge, USA, plasmid # 14390) was used as the expression vector. In this study, four DNA fragments including pAG vector, *C4H* gene, ADH1 terminator, and TEF1 promoter fragments were assembled using isothermal reaction of NEBuilder® HiFi DNA Assembly (New England Biolabs, USA) to yield pAG-CAT (C4H-ADH1t-TEF1p) vector. The DNA fragments were joined together via 20 bp-overlapping sequences in respective individual primer sets designed using NEBuilder® Assembly Tool (nebuilder.neb.com). The Vector fragment was amplified from CYC1 terminator to GPD promoter sequences in the original pAG423GPD-ccdB-Cerulean plasmid vector using Polymerase Chain Reaction (PCR) amplification. Fragments of TEF1 promoter and ADH1 terminator were PCR amplified using pCEV-G3-Km (Addgene, Cambridge, USA, plasmid # 46817) as the DNA template. C4H Fragment was obtained by amplifying *P. minus* cDNA using *P. minus* transcriptome dataset (Loke et al. 2017, 2016). Products obtained from PCR reactions were purified and assembled in 1:5 vector: insert ratio by incubating with NEBuilder® HiFi DNA Assembly Master Mix for 1 h. The assembled fragments were subsequently transformed into *E. coli* Top10 competent cells using heat shock method (Sambrook et al. 1989). Colony PCR of *E. coli* transformants was performed using KOD FX Neo DNA polymerase (Toyobo, Japan) to confirm the assembly of the 4 fragments in *E. coli*. Positive transformants exhibiting corresponding PCR products were subcultured for plasmid isolation and verification of correct nucleotide sequences via DNA sequencing.

Plasmid Transformation in *S. cerevisiae*

The recombined pAG-CAT shuttle plasmid containing *C4H* gene was transformed into *S. cerevisiae* competent

cells using Frozen-EZ Yeast Transformation II Kit™ (Zymo Research, USA) following the kit instruction manual. Briefly, 5 µL of purified DNA sample was mixed with the frozen yeast competent cells and incubated at 30°C for 1 h with intermittent shaking in every 15 min. The mixture was resuspended with 2x YPD medium and incubated at 30°C overnight or for at least 1 h prior to spreading onto selective SD-His$^-$ agar plate. Transformed yeast were incubated for 2-3 days at 30°C and colony PCR was performed on selected yeast colonies to confirm the insertion of *C4H* gene in the yeast cells using KOD FX Neo DNA polymerase protocol (Toyobo, Japan). The *C4H* gene sequence from the yeast colony PCR was verified via DNA sequencing. All plasmids and strains employed in this study are listed in Table 1.

Results and Discussion

Rapid construction of yeast vector for expressing plant biosynthetic genes

In this study, we aimed to construct yeast vector for the expression of phenylpropanoid biosynthetic gene via one-step and seamless DNA assembly method. Insert DNA fragments were designed in tandem with yeast regulatory elements for the creation of double expression cassettes under the control of strong constitutive GPD and TEF1 promoters. To express phenylpropanoid biosynthetic pathway in yeast, the rate-limiting enzyme cinnamate 4-hydroxylase encoded by *C4H* gene was cloned in the first expression cassette. As illustrated in Figure 1, the cloning of *C4H* gene was designed to be located in between the original GPD promoter and newly added ADH1 terminator in the pAG vector. Another promoter, TEF1p was also cloned upstream to the original CYC1 terminator in the pAG423GPD vector (Alberti et al. 2007).

Consequently, this arrangement enables the construction of the second expression cassette by additional cloning of desired coding sequences (CDS) in between the TEF1 promoter and CYC1 terminator. By transforming this pAG-CAT vector in *S. cerevisiae*, the *C4H* gene will be constitutively expressed. The expression of cytochrome P450-type C4H enzyme will enable the biosynthesis of plant phenolic compound, p-coumaric acid from trans-cinnamic acid under the control of the GPD promoter and ADH1 terminator.

One-step assembly of multiple DNA fragments via isothermal DNA assembly

For rapid yeast plasmid construction, we carried out homology-based DNA assembly of four DNA fragments to yield pAG-CAT yeast vector. The *in vitro* DNA assembly was performed by the enzymatic reactions of 5' exonuclease, DNA polymerase and DNA ligase that joined the fragments based on 15-25 bp overlapping regions between adjacent fragments. In this study, three insert fragments, namely C4H, ADH1 and TEF1, were designed to contain 20 bp overlapping sequences to adjacent fragments and between the insert fragments and vector. For DNA propagation in recombinant yeast, a shuttle plasmid pAG423GPD containing 2 µ origin of replication and Histidine auxotrophic marker was employed for the isothermal cloning-based recombination.

Figure 2 depicts the overall construction process of pAG-CAT recombined vector using isothermal assembly method. The C4H fragment with 1.5 kb size was obtained via PCR amplification of *P. minus* cDNA. The yeast ADH1 terminator and TEF1 promoter fragments respectively with PCR product sizes of 0.3 and 0.4 kb, were amplified using pCEV-G3-Km yeast vector as the DNA template. Assembly of the four DNA fragments (1 vector, 3 insert fragments) was verified using colony PCR and DNA sequencing PCR

TABLE 1. List of microbial strains and plasmids used in this study

Strain or plasmid	Genotype or construct	Reference or source
Bacterial strains		
Escherichia coli TOP10	F– *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara leu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	Invitrogen[a]
S. cerevisiae CEN.PK 2-1C	MAT**a**; *his3D1; leu2-3_112; ura3-52; trp1-289; MAL2-8c; SUC2*	EUROSCARF[b]
Plasmids		
pAG423GPD-ccdB-Cerulean	*E. coli-S. cerevisiae* shuttle vector, Cm^R, Amp^R His3 auxotrophic marker	Alberti et al. (2007)
pCEV-G3-Km	*E. coli-S. cerevisiae* shuttle vector, Amp^R, G418^R	Vickers et al. (2013)
pAG-CAT	pAG423GPD-ccdB-Cerulean containing *C4H* gene, ADH1 terminator, TEF1 promoter fragments	This study

Amp, ampicillin; Cm, chloramphenicol; R, resistance
[a]Invitrogen Corporation, Carlsbad, CA, USA
[b]European *Saccharomyces cerevisiae* Archive for Functional Analysis (EUROSCARF), Frankfurt, Germany

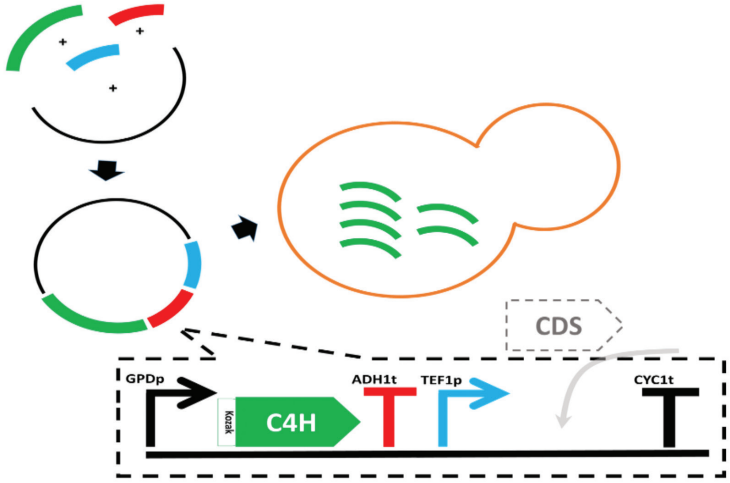

FIGURE 1. A simplified scheme for *in vitro* DNA assembly and construction of yeast plasmid with double expression cassettes. Three insert fragments (*C4H* gene, ADH1 terminator and TEF1 promoter) were assembled with linear yeast plasmid fragment in *E. coli* via isothermal reactions. The *C4H* gene from *P. minus* will be expressed heterologously in recombinant *S. cerevisiae* under the control of GPD promoter in the first expression cassette. Additional coding sequences (CDS) can be cloned in the second expression cassette under the control of TEF1 promoter

products from the *E. coli* transformants. A total PCR product size of 2.3 kb was obtained when using C4H forward and TEF1 reverse primers. This demonstrates the successful assembly of the insert and vector fragments in *E. coli*. The recombinant pAG-CAT (C4H-ADH1t-TEF1p) plasmid with the size of 8.9 kb attained after a single restriction enzyme digestion further confirmed the complete recombination of the three insert fragments with the constructed plasmid vector using our isothermal assembly strategy.

Overall, three DNA fragments were successfully assembled with vector in a single day and overall process requires a total of 3-5 days for DNA preparation and verification of assembled DNA sequences. This method is highly useful for the one-step and simultaneous construction of multiple 4-6 fragments as compared to traditional restriction-ligation cloning methods that involved multiple sequential steps that are time-consuming, laborious and limited by the availability of restriction enzyme sites (Chen 2016; Wang et al. 2012). In particular, co-expression of enzymes using restriction enzyme sites would require individual plasmid construction prior to obtaining the final multigene plasmid construct (Busso

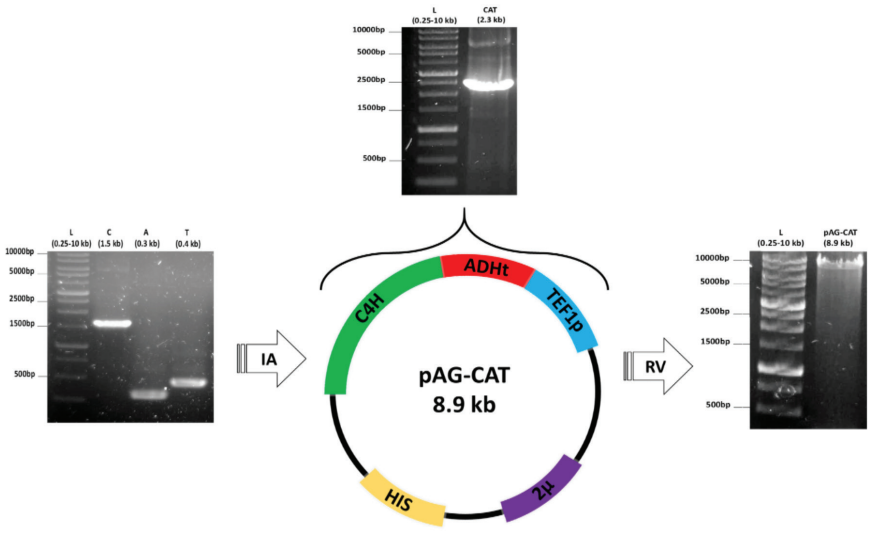

FIGURE 2. Overall construction of pAG-CAT plasmid using isothermal assembly method. Inset figures depicted electrophoresis gel results of respective DNA fragments. Lane C, A, T and CAT represent PCR products of C4H, ADHt, TEF1p and assembled C4H-ADHt-TEF1p fragment, respectively. Lane pAG-CAT indicates the total size of pAG-CAT vector linearized with restriction enzyme digestion. Lane L represents DNA ladder (ExcelBand™ 1 KB (0.25-10 kb) DNA Ladder; SMOBIO, Taiwan). IA and RV denote for isothermal assembly and recombined vector, respectively

et al. 2011; Ramzi et al. 2015). The employment of this homology-based assembly approach and other *in vitro* assembly techniques would further aid in the rapid construction of large constructs, particularly for complex biosynthetic pathways of plant metabolites and antibiotics (Luo et al. 2016).

TRANSFORMATION OF YEAST WITH PHENYLPROPANOID BIOSYNTHETIC GENE FROM *P. MINUS*

S. cerevisiae has been widely used for the expression of eukaryotic proteins and production of plant-derived metabolites. In this study, we aimed to establish yeast as the cloning and production host of phenylpropanoid and flavonoid biosynthetic pathways based on previous findings of *P. minus* (Ahmad et al. 2014; Loke et al. 2017) stem and roots. To this end, the cytochrome P450-type *C4H* gene from *P. minus* was amplified and assembled in the pAG-CAT shuttle vector for the plasmid transformation in *S. cerevisiae* CEN.PK 2-1C strain. Yeast transformants were selected for colony PCR analysis using C4H primer and the presence of *C4H* gene was confirmed by the successful amplification and DNA sequencing of the 1.5 kb PCR products (Figure 3). Similar 1.5 kb PCR product size was obtained in control of *C4H* gene fragment as a template. All yeast colonies that were randomly chosen exhibited the presence of heterologous *C4H* gene indicating viable propagation of the C4H-containing pAG-CAT vector in the recombinant *S. cerevisiae*.

C4H gene is considered the key enzyme in the phenylpropanoid and flavonoid biosynthesis in plant and engineered microbes (Koopman et al. 2012; Trantas et al. 2009). The engineered *S. cerevisiae* with this rate-limiting enzyme in the phenylpropanoid biosynthesis pathway would therefore serve as a starting platform for further assembly of other flavonoid biosynthetic genes. Compared to *E. coli*, the major advantage of using *S. cerevisiae* as the transformation host is the capacity of yeast to support the expression of membrane-bound cytochrome P450 family that is relatively difficult in *E. coli* (Leonard & Koffas 2007; Siddiqui et al. 2012). Furthermore, genetically-engineered *S. cerevisiae* can be further developed for the heterologous expression and production of plant-derived aromatic compounds owing to its innate capability to support aromatic amino acid metabolism and established genetic tools for efficient overproduction of metabolites that are naturally found in very small amounts (Siddiqui et al. 2012).

Importantly, an additional gene can be cloned in the second expression cassette under the control of the TEF1 promoter and CYC1 terminator. Upstream gene such as phenylalanine ammonia lyase (PAL) can be expressed for supplying the trans-cinnamate precursor in the recombinant yeast (Yan et al. 2005). Similarly, the catalytic activity of cytochrome P450 C4H can be further improved by co-expressing activating cytochrome P450 reductase (*CPR*) gene in the second expression cassette (Koopman et al. 2012). Another key enzyme in the phenylpropanoid biosynthetic pathway, 4-coumarate:CoA ligase (4CL) may also be cloned in the second expression cassette for the biosynthesis of p-Coumaroyl-CoA, an essential precursor for flavonoid and stilbenoid pathway (Jiang et al. 2005; Trantas et al. 2009).

CONCLUSION

Rapid construction of yeast expression cassettes for phenylpropanoid biosynthesis was successfully demonstrated in the recombinant *E. coli* and *S. cerevisiae*. By using this homology-based approach, multiple gene assembly of complex biosynthetic pathway genes can be achieved in shorter time due to the simplicity of the overlapping DNA fragment designs without the constraints form restriction-based cloning. This report will provide the basis to further engineer *S. cerevisiae* for the production of flavonoids and phenolic compounds using metabolic engineering and synthetic biology approaches.

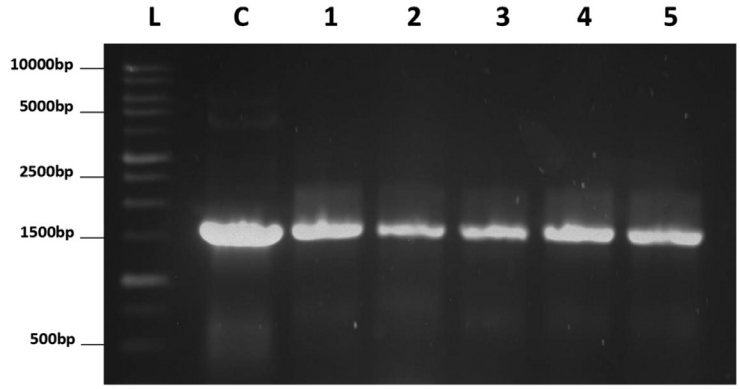

FIGURE 3. Colony PCR results of *C4H* gene in recombinant *S. cerevisiae* cells. Lane 1 to 5 represent specific PCR product of *C4H* gene in yeast cells that were randomly picked. The *C4H* gene sequence from the yeast colony PCR was verified via DNA sequencing. Lane C denotes PCR product of control sample using C4H fragment from *P. minus* as the template. Lane L indicates DNA ladder (ExcelBand™ 1 KB (0.25-10 kb) DNA Ladder; SMOBIO, Taiwan)

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