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Improved cloning and expression of cytochrome P450s and cytochrome P450 reductase in yeast

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Abstract

Combination of the pYeDP60 yeast expression system with a modified version of the improved uracil-excision (USER™) cloning technique provides a new powerful tool for high-throughput expression of eukaryotic cytochrome P450s. The vector presented is designed to obtain an optimal 5′ untranslated sequence region for yeast (*Kozak* consensus sequence), and has been tested to produce active P450s and NADPH–cytochrome P450 oxidoreductase (CPR) after 5′ end silent codon optimization of the cDNA sequences. Expression of two plant cytochrome P450s, *Sorghum bicolor* CYP79A1 and CYP71E1, and *S. bicolor* CPR2 using the modified pYeDP60 vector in all three cases produced high amounts of active protein. High-throughput functional expression of cytochrome P450s have long been a troublesome task due to the workload involved in cloning of each individual P450 into a suitable expression vector. The redesigned yeast P450 expression vector (pYeDP60u) offers major improvements in cloning efficiency, speed, fidelity, and simplicity. The modified version of the USER™ cloning system provides great potential for further development of other yeast vectors, transforming these into powerful high-throughput expression vectors.

Keywords: Cytochrome P450; Cytochrome P450 reductase; High-throughput heterologous expression; pYeDP60; Uracil-excision cloning; Yeast

Cytochrome P450 monooxygenases (P450s)[1] constitute a ubiquitous superfamily found in all eukaryotes and many prokaryotes [1,2]. In order to characterize the enzymatic activity of a particular P450 protein and to exploit these catalytic abilities for e.g., targeted metabolic engineering of desired natural product profiles, heterologous expression of the P450 protein is an essential step.

The first heterologous expression of a recombinant functional mammalian P450 was achieved in *Saccharomyces cerevisiae* [3]. Subsequently, other expression systems using bacteria or cell cultures from mammals or insects have been developed. Mammalian cells appear as the preferred system for expression of mammalian P450s for drug and carcinogenesis research. Expression of plant P450s have mostly been accomplished in yeast, bacteria or insect cells with yeast being the most frequently used system (reviewed in [4,5]).

The clear preference of yeast as heterologous expression host for eukaryotic P450s reflects (1) the engineering advantages associated with microorganisms, low media costs, high efficiency, and rapid growth in comparison with those of a higher eukaryotic host, (2) the availability of vectors with high yielding galactose-inducible GAL10-CYC1 hybrid promoters, (3) the availability of host strains which over-express different appropriate P450 reductase genes, and (4) the presence of an ER membrane environment. Furthermore, the choice of yeast as expression host affords the possibility to express active full-length P450s, whereas bacterial expression has often been dependent on truncation or substitution

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[1] *Abbreviations used:* P450s, P450 monooxygenases; CPR, NADPH-cytochrome P450 oxidoreductase; MCS, multiple cloning site; tyr, tyrosine; *At*ATR, *Arabidopsis thaliana* NADPH-cytochrome P450 oxidoreductase.

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of the N-terminal in order to achieve expression [6,7]. However, different expression systems may fulfill different objectives, and the choice of method must therefore be carefully considered according to the challenge faced. Difficulties experienced in expressing monocot plant P450 cDNAs in yeast have been overcome by partial recoding of the 5′ end. Monocot plants are GC rich with the third codon position often being G/C instead of the preferred A/T for yeast. Empirical silent recoding of rare yeast codons located close to the translation initiation site has significantly lowered the risk of translation failure [8,9].

The yeast expression vector pYeDP60 [10] has so far been the most successfully used for cytochrome P450 (reviewed in [5]). The multiple cloning site (MCS) in this vector offers a very limited degree of freedom with respect to the choice of restriction sites for cloning. This complicates the cloning process in cases where no optimal restriction sites can be included in the primers amplifying the sequence of interest. Repetition of restriction sites between the MCS and the sequence of interest often results in a prior need for either silent site-directed mutagenesis or partial digestions of the sequence that needs cloning.

The recent development of an improved uracil-excision based (USER™) cloning technique [11] provided a unique possibility to redesign the pYeDP60 vector in a manner that circumvents the above mentioned problems. The new high-throughput expression vector, designated pYeDP60u, contains an optimal yeast 5′ untranslated region (5′ UTR), and combines the advantages of pYeDP60 with the simplicity, speed, high efficiency and high fidelity of the improved uracil-excision system. Expression of two plant cytochrome P450s, *Sorghum bicolor* CYP79A1 and CYP71E1 as well as *S. bicolor* NADPH–cytochrome P450 oxidoreductase (*Sb*CPR2) using pYeDP60u in all three cases provided high amounts of active protein. The combination of the well established yeast-pYeDP60 expression system with the USER™ cloning technique provides a new powerful tool for high-throughput expression of eukaryotic P450s.

Materials and methods

Yeast strains, growth, and preparation of microsomes

Saccharomyces cerevisiae WAT11, a derivative of the W303-B strain (*MAT a*; *ade* 2-1; *his* 3-11, -15; *leu* 2-3, 112; *ura* 3-1; *can*^{R}; *cyr*$^{+}$) [10] expressing the *Arabidopsis thaliana* NADPH–cytochrome P450 oxidoreductase 1 (*At*ATR1) was used as host strain for expression of *Sb*CYP79A1 and *Sb*CYP71E1 expression. *Saccharomyces cerevisiae* W303-1A (*MAT a*; *ade* 2-1*; can* 1-100; *ura* 3-1; *leu* 2-3, 112 *his* 3-11, 15 *trp* 1-1; *ybp* 1-1) was used for expression of *Sb*CPR2. *Saccharomyces cerevisiae* transformations were performed by the improved lithium acetate procedure [12] with minor modifications. The amount of vector DNA used for each transformation reaction varied between 1 and 10 μg. Single stranded salmon sperm DNA (100 μg) was included as carrier. Growth and induction of cultures of WAT11 and W303-1A as well as preparation of microsomes were performed as previously reported [10], except that precultures from colonies were initiated in 5 mL SGI.

Construction of a pYeDP60 derived USER compatible vector

A chemically synthesized oligonucleotide cassette was ligated into the pYeDP60 vector following digestion with BamHI/EcoRI to generate the USER compatible vector, pYeDP60u. The cassette was constructed from two complimentary oligonucleotides (forward strand: 5′-phos-GATC CGCTGAGGATTAATTAAGCGGCCGCTTAATTAA CCCTCAGCG-3′ and reverse strand: 5′-phos-AAT TCGCTGAGGGTTAATTAAGCGGCCGCTTAATTA ATCCTCAGCG-3′), which were combined and diluted to 10 μM, incubated at 90 °C for 5 min, and allowed to cool to room temperature. The pYeDP60u vector was verified by sequencing.

Cloning of SbCPR2

The full-length mRNA sequence of a *SbCPR* was kindly provided by B.A. Halkier. The sequence was identical to the transcript of the gene Sbi_0.43436 (prediction of putative *CPR* gene based on the partial EST sequence, EST: BM326206) in the Phytozome.net *S. bicolor* genome database. Three different CPR sequences are registered in the genome database. Sequence alignments of this *Sb*CPR with CPR sequences from other plant species demonstrated that the *S. bicolor* sequence showed highest similarity to isoform 2 (CPR2). Based on annotations in the genome and EST databases, multiple sequence alignments with other closely related plant CPRs, and on comparison of hydrophobicity profiles, the most upstream of three putative start codons was chosen as the most likely codon for translation initiation. The *SbCPR2* cDNA was then amplified directly from a custom made *S. bicolor* cDNA library made from 1 to 2 cm high *S. bicolor* (L.) Moench etiolated seedlings [13] using PCR with the primers shown in Table 1.

Insertion of PCR fragments into pYeDP60u

The pYeDP60u vector was prepared according to Nour-Eldin et al. [11]. To enable ligation into the pYeDP60u vector, all forward and reverse PCR primers contained a tail of 9 and 8 nt, respectively (compare Fig. 1). The linkers added were: forward 5′-GG**A**TTAA**U** + A + sequence of coding strand of target DNA; reverse 5′-GG**G**TT AA**U** + optimized stop codon (TAA) + sequence complimentary to coding strand of target DNA. *SbCYP79A1*, *SbCYP71E1*, and *SbCPR2* were all recoded at the 5′ end (Table 1). *SbCYP71E1* was also recoded at the last 5 codons at the 3′ end (Table 1). PCR was performed using PfuTurbo® C_x Hotstart DNA polymerase (Stratagene) according to manufacturer's instructions. To avoid