Chapter 4

Preparation of *Saccharomyces cerevisiae* Expression Plasmids

David Drew and Hyun Kim

Abstract

Expression plasmids for *Saccharomyces cerevisiae* offer a wide choice of vector copy number, promoters of varying strength and selection markers. These expression plasmids are usually shuttle vectors that can be propagated both in yeast and bacteria, making them useful in gene cloning. For heterologous production of membrane proteins, we used the green fluorescent protein (GFP) fusion technology which was previously developed in the *Escherichia coli* system. We designed an expression plasmid carrying an inducible *GAL1* promoter, a gene encoding a membrane protein of interest and the GFP-octa-histidine sequence. Here we describe construction of multi-copy yeast expression plasmids by homologous recombination in *S. cerevisiae*.

Key words: Green fluorescent protein, Galactokinase, Fluorescence

Abbreviations

GAL	Galactokinase
GPD	Glyceraldehyde-3-phosphate dehydrogenase
TEF	Translation elongation factor 1α
TEV site	Tobacco etch virus protease cleavage site
yEGFP	Yeast-enhanced green fluorescent protein

1. Introduction

One of the advantages working with *S. cerevisiae* is that there is a wide selection of vectors that can be used for various purposes. These vectors were developed and modified during the 1990s and have been continuously optimised for a range of applications.

Roslyn M. Bill (ed.), *Recombinant Protein Production in Yeast: Methods and Protocols*, Methods in Molecular Biology, vol. 866, DOI 10.1007/978-1-61779-770-5_4, © Springer Science+Business Media, LLC 2012

Usually they are shuttle vectors that can be transferred between yeast and bacteria, making them useful tools for gene cloning and molecular biology in general.

Yeast vectors are roughly divided into two groups: one with a yeast centromere sequence (*CEN*) and the other with a 2μ origin of replication. The former is a mitotically stable yeast replicating plasmid with only a single copy present per cell (1); whereas 2μ plasmids have a copy number of about 20 per cell (2). 2μ plasmids are generally more useful for heterologous protein production.

For regulated or constitutive production of heterologous proteins, Mumberg et al. tested various promoters in both the *CEN* and the 2μ plasmids (3, 4). They showed that the galactokinase (*GAL1*) promoter rendered the highest gene expression for controlled and inducible expression of heterologous genes (3). Among the tested constitutive promoters, promoters derived from genes of glyceraldehyde-3-phosphate dehydrogenase (*GPD*) and translation elongation factor 1α (*TEF*) exhibited high strength in heterologous gene expression (4). For both cases, yeast cells carrying vectors of the pRS420 series (2μ plasmids) showed higher protein yields than those containing the pRS410 series (*CEN* plasmids). To further optimise these vectors, they were constructed with four different selection markers, *HIS3*, *TRP1*, *LEU2* or *URA3* (3, 4). Thus, they offer a choice of copy number, promoter and selection marker, so that researchers can choose an expression plasmid according to their needs. A complete set of these expression vectors can be found and purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA).

The heterologous production of membrane proteins has been a challenging task as membrane proteins are often produced in low yields and/or identification of high-yielding systems is time-consuming. To facilitate identification of high-yielding conditions, we took advantage of the green fluorescent protein (GFP) fusion approach that was previously used in *Escherichia coli* (5). It was shown that GFP was fluorescent only when GFP-tagged membrane proteins were correctly targeted to the plasma membrane. If they were aggregated in the cytosol, there was no fluorescent signal. The fluorescence resulting from recombinant membrane protein–GFP fusions could be easily measured both in liquid culture and in standard SDS gels.

We designed an expression vector carrying either constitutive, *TEF*, or regulated *GAL1* promoters in a 2μ plasmid (pRS426) with C-terminal GFP and an octa-histidine tag for detection and purification of proteins (6). A yeast codon optimised Tobacco Etch Virus (TEV) protease site was designed upstream of GFP for removal of the GFP-His_8 tag.

When we initially tested production levels of more than 20 membrane proteins using the two vectors above in *S. cerevisiae*, we found that the *GAL1* promoter gave overall higher expression than the constitutive *TEF* promoter (6). Further, we found that

membrane yields were enhanced in the *pep4* deletion strain in which the vacuolar endopeptidase Pep4p is deleted. Deletion of the *pep4* gene not only inhibits Pep4p protease activity but also reduces the levels of other vacuolar hydrolases (7).

Here we describe the practical steps in using homologous recombination to generate sequences encoding a membrane protein–GFP-His_8 tag fusion in the 2μ yeast vector carrying a *GAL1* promoter, pRS426GAL1. This strategy has been successfully employed by others to overproduce eukaryotic membrane proteins (8).

2. Materials

1. Yeast *pep4* deletion strain, FGY217 (*MATa*, *ura3-52*, *lys2Δ201*, *pep4Δ*) (9).
2. A vector containing the yeast-enhanced green fluorescent protein (pUG35) (10).
3. Growth medium without uracil (for 1 L, 6.7 g yeast nitrogen base without amino acids (BD Difco, cat. No. 291920)), 2 g yeast synthetic drop-out medium supplement without uracil (Sigma, cat. No. Y1501), and either 2% glucose (for pre-culture) or 0.1% glucose (for expression culture). For plates, add 20 g of bacto agar (Sigma, cat No. A5306). D-(+) glucose can be purchased from Sigma (cat. No. G7021).
4. 20% galactose (w/v) (Sigma, cat. No. G0625).
5. *SmaI* restriction enzyme (Invitrogen, cat. No. 15228018).
6. For yeast transformation with lithium acetate, YPD medium (BD Difco, cat. No. 242810), polyethylene glycol (PEG) 3350 (Sigma, cat. No. P3640), lithium acetate (Sigma, cat. No. L4158), salmon sperm DNA (Sigma, cat. No. D1626), dimethyl sulfoxide (DMSO) (Sigma, cat. No. D2438) are needed.
7. PCR reagents (polymerases and dNTPs are available from various companies).
8. Mini-prep plasmid kit (Qiagen).
9. Acid-washed glass beads, 500 μm (Sigma).

3. Methods

3.1. Construction of an Expression Vector Carrying the TEV Site-GFP-His_8 Sequence

1. Amplify a fragment containing the yeast-enhanced GFP sequence (yEGFP) excluding the start codon from the pUG35 plasmid (10) by PCR using two primers: forward 5′-TCTAGAACTAGTGGATCCCCCCCCGGGGAAAATT TATATTTTCAAGGTC-3′ and reverse 5′-GACGGTATCGAT AAGCTTGATATCAATTCCTGCAGTTAATGATGATG-3′.

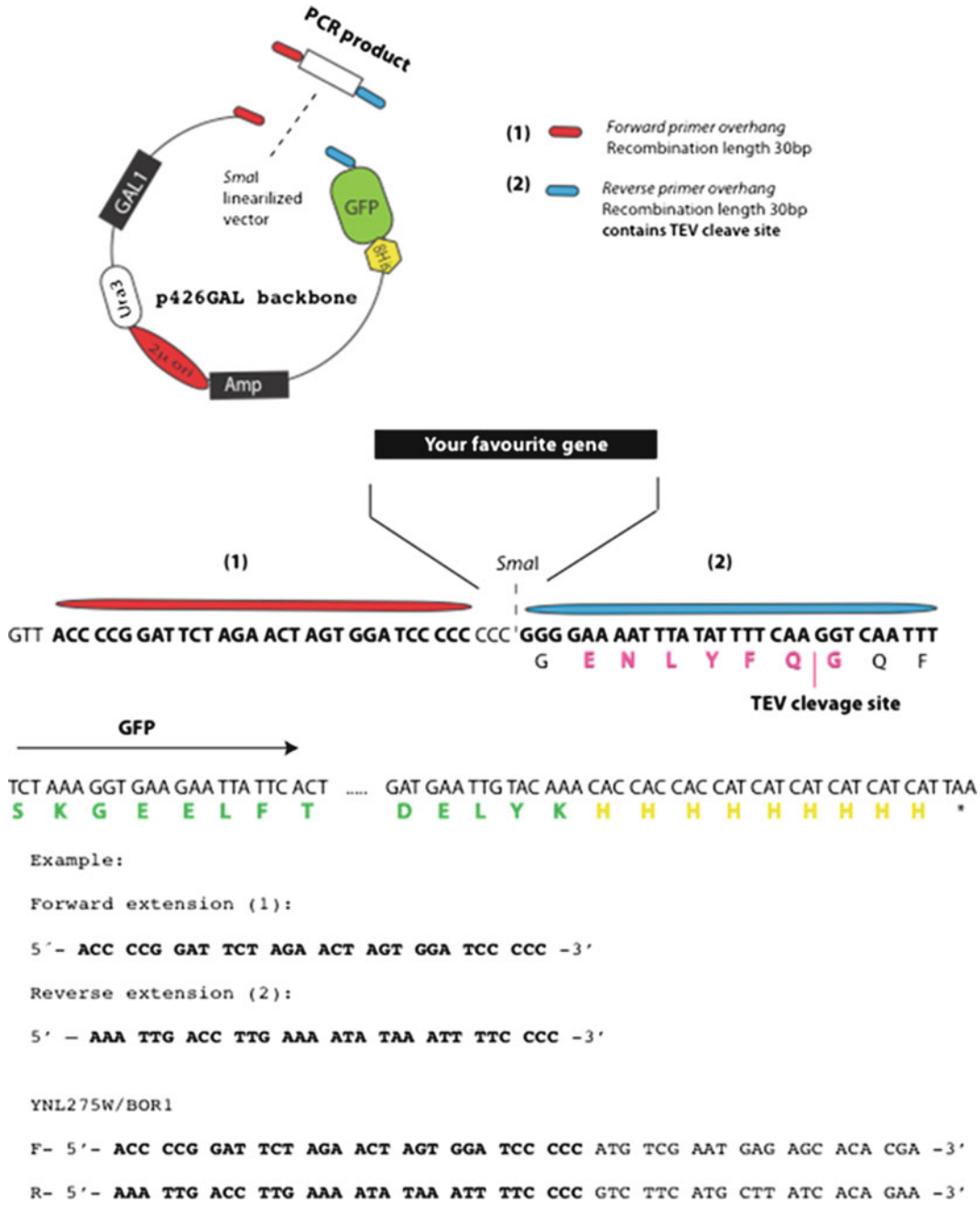

Fig. 1. Cloning by homologous recombination into a 2μ *S. cerevisiae* GFP-fusion expression vector (11). Primer design for the gene YNL275W/BOR1 is shown as an example. The sequences in *bold* are the homologous recombination sites to the vector.

These primers contain sequences encoding (1) tobacco etch virus (TEV) cleavage sequence (ENLYFQG/S), (2) *SmaI* endonuclease restriction site, (3) octa-histidine tag on the reverse primer and (4) homologous recombination sites to the pRS426 plasmids (Fig. 1) (11).

2. Digest pRS426GAL1 with a *SmaI* restriction enzyme to linearise the vector (see Note 1).
3. Transform any uracil auxotrophic *S. cerevisiae* strain with the PCR-amplified fragment and the *SmaI*-digested pRS426GAL1 plasmid using lithium acetate (12). Select on medium lacking uracil. To assess the number of background colonies carrying only an empty vector, prepare one sample lacking the PCR product. The number of these background colonies should be very low.
4. Inoculate yeast transformants in 5 mL of medium lacking uracil, grow overnight at 30°C and isolate plasmids by following the mini-prep plasmid kit manufacturer's protocol with the exception that the harvested cells should be suspended in the kit's resuspension buffer (the first buffer in the mini-prep kit). Then, add an equal volume of acid-washed glass beads to the cell suspension, vortex at maximum speed for 5 min, collect the glass beads and unbroken cells with a brief centrifugation step and transfer the supernatant to a new tube. Thereafter proceed to the next step of the mini-prep kit protocol. These plasmids should be further amplified and isolated from bacterial cells for DNA sequencing. The pRS420 series of vectors contains an ampicillin-resistance marker for selection in bacterial cultures.
5. Isolate plasmids from bacterial cells and sequence to confirm an in-frame TEV site-GFP-His_8 sequence. pRS426GAL1 carrying the TEV-site-GFP-His_8 sequence is designated pDDGFP-2 (6).

3.2. Cloning of Membrane Protein-Encoding Gene(s) into the pDDGFP-2 Plasmid

1. Amplify the gene of interest with primers that include 5′ overhangs (35 bp) complementing the upstream and downstream sequences to either side of the *SmaI* site in the pDDGFP-2 vector (Fig. 1) (11).
2. Carry out transformation of FGY217 (*MATa*, *ura3-52*, *lys2Δ201*, *pep4Δ*) with the PCR-amplified gene and *Sma*I-linearised pDDGFP-2 (9) using lithium acetate (11, 12) and select on medium lacking uracil. As for the pDDGFP-2 vector above, prepare one sample without the PCR product to assess the number of background colonies. Yeast plates can be stored at 4°C for at least 1 month without a reduction in production levels.
3. To confirm that a colony harbours the vector and gene of interest, inoculate 5 mL of medium lacking uracil with the colony, grow overnight at 30°C and either do yeast colony PCR using sequencing primers or check the fluorescence of whole cell lysates (described in detail in Chapter 8).

4. Note

1. Digestion with *Sma*I gives rise to blunt ends that eliminate background colonies caused by re-annealing of a linearised vector by homologous recombination during transformation. There may be a higher number of background colonies when using restriction enzymes that produce sticky ends. This does not appear to be reduced by including a dephosphorylation step.

Acknowledgments

This work was supported by the Royal Society (United Kingdom) through a University Research Fellowship to DD and by a Basic Science Research Program grant through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (NRF0409-20100093) to HK.

References

1. Sikorski RS, Hieter P (1989) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics 122:19–27
2. Christianson TW, Sikorski RS, Dante M, Shero JH, Hieter P (1992) Multifunctional yeast high-copy-number shuttle vectors. Gene 110:119–122
3. Mumberg D, Muller R, Funk M (1994) Regulatable promoters of *Saccharomyces cerevisiae* – comparison of transcriptional activity and their use for heterologous expression. Nucleic Acids Res 22:5767–5768
4. Mumberg D, Muller R, Funk M (1995) Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. Gene 156:119–122
5. Drew DE, von Heijne G, Nordlund P, de Gier JW (2001) Green fluorescent protein as an indicator to monitor membrane protein overexpression in *Escherichia coli*. FEBS Lett 507:220–224
6. Newstead S, Kim H, von Heijne G, Iwata S, Drew D (2007) High-throughput fluorescent-based optimization of eukaryotic membrane protein overexpression and purification in *Saccharomyces cerevisiae*. Proc Natl Acad Sci USA 104:13936–13941
7. Woolford CA, Daniels LB, Park FJ, Jones EW, Van Arsdell JN, Innis MA (1986) The *PEP4* gene encodes an aspartyl protease implicated in the posttranslational regulation of *Saccharomyces cerevisiae* vacuolar hydrolases. Mol Cell Biol 6:2500–2510
8. Li M, Hays FA, Roe-Zurz Z, Vuong L, Kelly L, Ho CM, Robbins RM, Pieper U, O'Connell JD 3rd, Miercke LJ, Giacomini KM, Sali A, Stroud RM (2009) Selecting optimum eukaryotic integral membrane proteins for structure determination by rapid expression and solubilization screening. J Mol Biol 385:820–830
9. Kota J, Gilstring CF, Ljungdahl PO (2007) Membrane chaperone Shr3 assists in folding amino acid permeases preventing precocious ERAD. J Cell Biol 176:617–628
10. Cormack BP, Bertram G, Egerton M, Gow NA, Falkow S, Brown AJ (1997) Yeast-enhanced green fluorescent protein (yEGFP): a reporter of gene expression in *Candida albicans*. Microbiology 143:303–311
11. Drew D, Newstead S, Sonoda Y, Kim H, von Heijne G, Iwata S (2008) GFP-based optimization scheme for the overexpression and purification of eukaryotic membrane proteins in *Saccharomyces cerevisiae*. Nat Protoc 3:784–798
12. Amberg DC, Burke DJ, Strathern JN (2005) Methods in yeast genetics: a cold spring harbor laboratory course manual. Cold Spring Harbor Laboratory Press, New York, USA.