A novel strategy for constructing N-terminal chromosomal fusions to green fluorescent protein in the yeast *Saccharomyces cerevisiae*

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Abstract A novel rapid polymerase chain reaction (PCR)-based technique for N-terminal attachment of green fluorescent protein (GFP) to a yeast protein is described. Genomic integration of a PCR-generated *loxPkanMX4loxP*-yEGFP fusion cassette immediately upstream of the open reading frame (ORF) allows for selection of G418 resistant transformants carrying GFP fused N-terminally to the protein of interest. In a subsequent step, the *loxPkanMX4loxP* selection marker that is inserted between the tagged ORF and the endogenous promoter is excised upon site-specific recombination between the loxP sites by Cre recombinase, leaving behind in the promoter one loxP site, immediately upstream of the GFP start codon. The essential protein Ydl193wp of unknown function and the oleate-inducible fatty acid activation protein, encoded by *FAA2*, were N-terminally tagged using the novel technique. Both experiments yielded viable haploid strains with growth phenotypes indistinguishable from the wild type strain. The subcellular localization pattern for the chromosomally expressed GFP-Ydl193wp to the endoplasmic reticulum and lipid particles was identical to the pattern observed for a plasmid-borne GFP construct expressed under control of the *MET25*^{p} promoter, albeit at a lower level and with a more homogeneous distribution among the cell population. GFP-*FAA2* was inducible by oleate, as is the wild type gene, demonstrating that specific expression patterns are not grossly affected by the promoter manipulation. In agreement with previous reports, GFP-Faa2p was found to localize to peroxisomes.

Key words: Green fluorescent protein; N-terminal fusion; loxP/Cre recombinase; Genomic integration; Homologous recombination

1. Introduction

Green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* is an attractive tool to study biological processes in living cells. Numerous cloning vectors have been developed for N- and C-terminal GFP attachment in yeast (for review see [1]). GFP fusion gene expression from episomal plasmids is commonly driven by heterologous promoters such as *ADH1* (constitutive) or *GAL1/10* (galactose-inducible), *MET25* (induction on methionine-free medium) or *CUP1* (copper induction). Therefore, growth conditions have to be adjusted for induction, and specific expression patterns of the tagged gene are obscured. Furthermore, episomal plasmids require maintenance of selection pressure, limiting the observation of GFP fusions under various growth conditions. Typically, plasmid-borne GFP expression results in rather heterogeneous staining intensities in a cell population. Subcellular localization patterns such as targeting or retention sequences, N- or C-terminal post-translational modification sites or functional domains are limitations for amino- or carboxy-terminal tag attachment.

In yeast, due to the efficiency of homologous recombination, chromosomal tagging with GFP at the C- or N-termini of proteins [2–4] provides numerous advantages over conventional cloning on episomal plasmids (see [1] for review), namely ease of construction, stable integration and homogeneous labeling patterns among the cell population. Furthermore, in case of C-terminal fusions, expression patterns are more comparable to the authentic gene due to expression from the endogenous promoter. This approach is based on the polymerase chain reaction (PCR) amplification of a transformation cassette comprising GFP and a selectable heterologous marker (e.g. *kanMX* [5]), using long hybrid primers with tails of more than 50 nucleotides (nt) homologous to the desired integration site at the 3′-end of the reading frame, right before the stop codon. After transformation and homologous recombination, transformants can be selected based on the marker. Correct integration results in a C-terminal in-frame GFP fusion, whose expression is driven by the endogenous promoter. However, in some cases C-terminal fusions may not be feasible due to C-terminal modification of the tagged protein (e.g. GPI anchor attachment) or targeting sites that get masked by the GFP tag (e.g. -SKL sequence for peroxisomal localization). For N-terminal chromosomal fusions, usually a heterologous promoter is used to drive expression, obscuring endogenous expression levels and patterns. The major drawback of a PCR approach for N-terminal GFP attachment is that the insertion of the marker–GFP fusion cassette separates the endogenous promoter from the open reading frame (ORF) of the tagged gene. Thus, transformants are unable to properly initiate transcription of the respective gene, unless a heterologous or homologous promoter sequence is cloned into the GFP cassette, adding further cloning steps and obscuring the ease of a PCR-based fusion strategy.

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Abbreviations: PCR, polymerase chain reaction; CEN, centromere; GFP, green fluorescent protein; yEGFP, yeast (*Candida albicans*) optimized enhanced green fluorescent protein; nt, nucleotides; ORF, open reading frame

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We have created a novel *loxPkanMXloxP*-yEGFP (yeast (*Candida albicans*) optimized enhanced green fluorescent protein) fusion cassette as a PCR template for chromosomal N-terminal tagging with GFP in yeast, combining *kanMX*-based selection with subsequent removal of the marker by Cre/loxP-mediated marker excision. The Cre/loxP recombination system of bacteriophage P1 has been shown to mediate efficient recombination in *Saccharomyces cerevisiae* between directly repeated loxP sites flanking a marker gene, resulting in excision of the marker upon expression of the Cre recombinase [6]. This technique has also been used for repeated use of *kanMX* as a selection marker for multiple gene disruptions [7] or multiple immuno-tag attachment [8] in yeast. As test systems we have tagged the essential protein encoded by ORF YDL193w, demonstrating functional expression of the N-terminal chromosomal GFP fusion after marker excision. The oleate-inducible non-essential gene *FAA2* was used to demonstrate that functionality of the promoter was retained after marker excision.

2. Materials and methods

2.1. Strains and media

Escherichia coli strain XL1-Blue *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q}*ZΔM15* Tn*10* (Tet^{r})]^{c} (Stratagene) was used for plasmid maintenance and amplification. Plasmids pUG34 and pRN295 (both containing yEGFP), pUG6 (harboring *kanMX4* flanked by loxP sites) and pSH47 (ori, ampR; *CEN6/ARSH4*; *GAL1*^{p}-Cre-*CYC1*^{T}; *URA3*) were obtained from J. Hegemann (Düsseldorf, Germany; for details on the plasmids see: http://www.mips.biochem.mpg.de/proj/yeast/info/tools/hegemann/gfp.html).

Yeast strain FY1681 *Mata/Matα ura3-52/ura3-52 his3Δ200/his3Δ200 TRP1/TRP1 LEU2/LEU2* and plasmid pPK199 were provided by Peter Kötter (EUROSCARF, Frankfurt, Germany). Transformation of FY1681 with plasmid pPK199 (*MET25*^{P}-yEGFP(Nfus)-YDL193w *HIS3 CEN6/ARSH4*) yielded strain FY1681{pPK199}. The following strains were constructed in the course of this study: BPY37: *Mata ura3-52 leu2Δ1 trp1Δ63 his3Δ200*; BPY46: *Mata/Matα* yEGFPgN-YDL193w/yEGFPgN-YDL193w *ura3-52/ura3-52 his3-Δ200/his3Δ200 LEU2/LEU2 TRP1/TRP1*; BPY62: *Mata* yEGFPgN-*FAA2 ura3-52 leu2Δ1 trp1Δ63 his3Δ200*; BPY63: *Mata ura3-52 leu2Δ1 trp1Δ63 his3Δ200* {pKN102}. Strain YAJ8D *Mata pex3Δ::kanMX4 ura3-52 trp1Δ63 his3Δ200* was provided by A. Jandrositz.

Media: bacteria and yeast strains were grown in standard LB (5 g/l yeast extract (USB), 10 g/l tryptone (Difco), 5 g/l NaCl (Merck)) or YPD (10 g/l yeast extract, 20 g/l peptone (Difco), 20 g/l glucose (Merck)) media, respectively. Geneticin resistant yeast colonies were selected on YPD/G418 medium (YPD+200 mg/l geneticin (G418); Calbiochem). Yeast cells transformed with plasmid pSH47 were selected and maintained on uracil-free medium (6.7 g/l yeast nitrogen base (USB), 10 g/l glucose, supplemented with all amino acids and bases (Merck), except uracil); −ura/galactose (lacking uracil but containing 10 g/l galactose instead of glucose) was used for induction of the Cre recombinase. Ampicillin resistant *E. coli* transformants were selected on LBA (5 g/l yeast extract, 10 g/l tryptone, 5 g/l NaCl, 100 mg/l ampicillin (Amaresco)). Oleate media contained 6.7 g/l yeast nitrogen base, 10 g/l Brij58 (Sigma) and 1 g/l oleic acid (Merck). For solid media plates, 20 g/l agar (USB) was added.

2.2. Construction of plasmid pYGFPgN ('genomic N-terminal')

yEGFP was PCR-amplified from plasmid pUG34 with primers pEGFP-Nfus_direct (5′-ggcgatatcATGTCTAAAGGTGAAGAATTAT-3′) and pEGFP-Nfus_reverse (5′-ccactagtTTGTACAATTCATCCATACC-3′) containing *Eco*RV and *Spe*I restriction sites (underlined), respectively, at their 5′-ends. Capital letters delineate regions homologous to the respective template plasmids. The resulting 729 bp fragment was restricted with *Spe*I and *Eco*RV and ligated into plasmid pUG6 cleaved with the same restriction enzymes, resulting in plasmid pUG6-yEGFP.

For construction of the *kanMX4* marker flanked by reversed loxP sites, we amplified a 1545 bp < *loxPkanMX4* < *loxP* PCR product from plasmid pUG6 with primers loxP_up (5′-tcccccgggataacttcgtatagcatacattatacgaagttatCTAGAGATCTGTTTAGCTTGCCTC-3′; *Sma*I) and loxP_down (5′-tagccagctgataacttcgtataatgtatgctatacgaagttatCTCGAGAGCTCGTTTTCG-3′; *Pvu*II) carrying the respective restriction sites at their 5′-ends. The *Sma*I/*Pvu*II-cleaved fragment was cloned into the respective sites of plasmid pUG6-yEGFP, replacing the original > *loxPkanMX4loxP* > fragment. The redesign of the loxP recombination sites was necessary to minimize their core sequence and to avoid any potential translation initiation within these sites since one of them would be left behind in the promoter sequence of the tagged gene after *kanMX4* marker excision. In the final construct, termed pYGFPgN, the loxP sites were reduced to a 34 bp core sequence, in addition to 6 bp introduced by the cloning procedure between the loxP site and the ATG of the GFP coding sequence. The modified parts of the resulting plasmid pYGFPgN were sequenced to verify correct insertion of the yEGFP and *loxPkanMX4-loxP* fragments.

2.3. PCR-mediated generation of the N-terminal yEGFP fusion cassette for chromosomal integration

The chimeric primers for PCR-mediated generation of the yEGFPgN fusion cassette typically consisted of a 70 nt 5′-segment homologous to a region directly upstream of the start ATG (forward primer), or to the sequence immediately downstream of and including ATG (reverse primer) of the gene of interest. The 3′-segments of the primers (12 and 24 nt, respectively) were identical for all yEGFP-tagged genes, either UPp (forward) homologous to the upstream loxP site, or DOWNp (reverse), homologous to the 5′-end of EGFP. The primer sequences for the tagged genes, YDL193w (primers #3 and #4) and *FAA2* (primers #5 and #6) are shown in Table 1. A typical PCR reaction of 25 μl contained 0.5 μM primers each, 1× Pwo DNA polymerase buffer with 2 mM $MgSO_4$ and 0.1 U/μl Pwo DNA polymerase, 200 μM dNTPs, 2 ng/μl pYGFPgN as the template. The PCR conditions were: denaturation at 94°C for 2 min; 10 cycles at 94°C for 30 s, 52°C 30 s, 72°C 2 min and 20 cycles at 94°C for 30 s, 72°C 150 s, followed by a final elongation step at 72°C for 10 min. The theoretical size of the PCR fragment harboring the *kanMX4* selection marker flanked by upstream and downstream loxP sites and

Table 1
Primers used in this study

Primer number	Name	Sequence (5′ → 3′)
1	UPp (3′-segment)	(70 nt upstream of ATG) – CGGCCGCCAGGG
2	DOWNp (3′-segment)	(70 nt downstream of ATG+ATG) – TTTGTACAATTCATCCATACCATG
3	YDL193w_loxPyEGFP up	attagttggcaaattacacaatttttttgatacaatatatatacatatatttttcccca-ttgtttttcttagCGGCCGCCAGGG
4	YDL193w_loxPyEGFP down	cgatctttctatgcggtttttcattaggggctccattgctttatcatccttttttg-atcatcgtgggcatTTTGTACAATTCATCCATACCATG
5	FAA2_loxPyEGFP up	aacgcatggctaagggaagtggaagaatgcaggttacaaaaaacggataag-aacaaacttgtttcgaaatCGGCCGCCAGGG
6	FAA2_loxPyEGFP down	ttgtcttcaaactttcgaaacgaggatccgattcaattaaatcggtaagtgca-taatctggagcggccatTTTGTACAATTCATCCATACCATG

Capital letters indicate sequences homologous to the respective template plasmids; small letters indicate sequences homologous to the respective chromosomal integration sites.

GFP is 2399 bp, including 70 bp homologous to the integration sites on either end.

2.4. Transformation of yeast strains and excision of the kanMX marker

Strains FY1681 and BPY37 were transformed with linear PCR fragments as described [9] and selected on YPD/G418 medium. In a second transformation step, plasmid pSH47 [7] was transformed into a geneticin resistant transformant and uracil prototrophic (Ura^+) transformants were selected on plates lacking uracil. Geneticin resistant and Ura^+ transformants were grown in −ura/galactose medium for at least 4 h to induce the Cre recombinase, as described [7]. Correct insertion into the chromosome and excision of the *loxP-kanMX4loxP* fragment were verified by diagnostic PCR with appropriate primers (data not shown).

2.5. Construction of yEGFP(Nfus)-FAA2

For construction of a plasmid harboring *FAA2* fused to GFP, under control of the $MET25^p$ promoter, the *FAA2* ORF was PCR-amplified from wild type genomic DNA (strain BPY37) using primers FAA2_forward (5′-gctctagagccgctccagattatgcac-3′) and FAA2_reverse (5′-gcgtcgacctaaagcttttctgtcttgactagtgaac-3′) that contained flanking *Xba*I and *Sal*I restriction sites (underlined). The PCR product was cut with these enzymes and cloned into the respective restriction sites of the GFP fusion vector pRN295. The resulting plasmid was termed pKN102.

2.6. Fluorescence microscopy

Cultivation of the various GFP fusion and respective control strains is outlined in detail in the Section 3. Cells were prepared for microscopic inspection as described [1]. Microscopy was performed on a Leica TCS 4D confocal microscope (100×/1.4 n.a. lens), using 488 nm excitation and a 525/50 band pass filter for GFP detection [1]. Transmission images were recorded using DIC optics.

3. Results and discussion

3.1. Strategy for the chromosomal integration of yEGFP at the 5′-end of an ORF of interest (Fig. 1)

3.1.1. Generation of the linear DNA fragment for integration into the genome. Plasmid pYGFPgN contains the *kanMX4* marker flanked by two tandemly arrayed loxP sites, together with a promoterless yEGFP that lacks a stop codon. By using chimeric primers UPp and DOWNp, PCR fragments are generated that contain *loxPkanMX4loxP*-yEGFP flanked by 70 nt homologous to the gene of interest. Primer UPp consists of a 3′-sequence that primes to 12 bp upstream of the first loxP site on the template plasmid pYGFPgN. Direct priming within the loxP site is avoided since this would lead to the generation of two different PCR fragments favoring the shorter reaction product, lacking the *kanMX4* gene. The 5′-sequence of primer UPp comprises 70 nt homologous to a region directly upstream of the start ATG of the gene of interest.

Primer DOWNp contains a 5′-segment that is complementary to the first 70 nt of the gene of interest (including the start codon) followed by a 3′-segment priming at the 3′-end of the yEGFP ORF. Sequences for UPp and DOWNp 3′-segments are invariable for various fusions and are shown in Table 1. Overall, the N-terminal fusion procedure results in an insertion of 53 bp between the authentic promoter sequence and the GFP start codon. Although an interference of the inserted sequence with promoter activities cannot be ruled out, data obtained in mammalian systems [10] and yeast (see below) support the feasibility of this approach. Furthermore, conventional cloning on episomal plasmids also introduces additional base pairs between the promoter sequence and the GFP reading frame, however, proper expression control usually is still maintained.

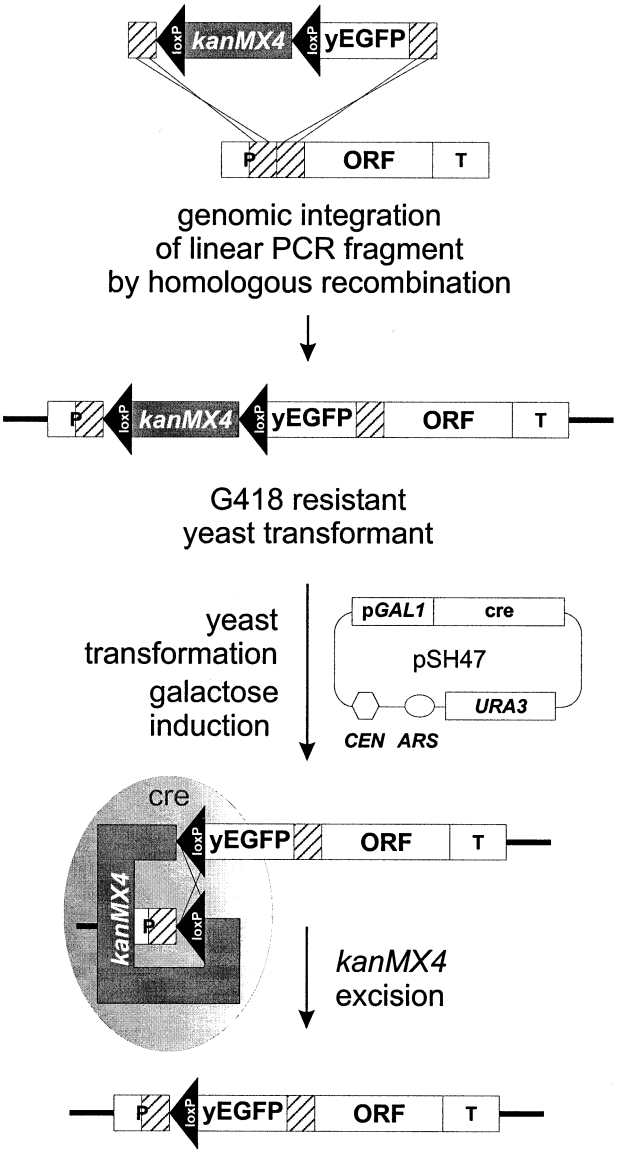

Fig. 1. Introduction of the linear PCR fragment into yeast and selection for geneticin (G418) resistant transformants. P, promoter; T, terminator. The linear PCR fragment generated with primers pUP and pDOWN is transformed into yeast and integrates into the genome by homologous recombination via the flanking 70 bp homology regions. Upon correct integration, the fragment is inserted in-frame directly upstream of the ORF and separating it from its endogenous promoter. Excision of the *kanMX4 marker gene. P, promoter; T, terminator; CEN, centromere; ARS, autonomous replication sequence; cre, Cre recombinase; pGAL1, GAL1^p promoter. Upon induction of the Cre recombinase, the kanMX4 marker sequence located between the tandemly arrayed loxP sites is excised. Cells are rendered sensitive to geneticin. Plasmid pSH47 is lost upon growth under non-selective conditions in the presence of 5-FOA.*

3.2. Genomic N-terminal yEGFP-tagging of YDL193w and FAA2

For the construction of the chromosomal N-terminal yEGFP fusions to YDL193w and *FAA2*, we basically followed the procedure described under Section 2. Depending on the induction time of the Cre recombinase (2–6 h), about 30–80% of the cells harboring the *loxPkanMX4loxP* fragment became G418 sensitive, indicative of loss of the *kanMX* marker.

YDL193w is an essential gene of unknown function and was previously identified to be a component of lipid particles in yeast [11]. Null mutations of YDL193w are lethal, thus, the gene was a suitable test system for the N-terminal tagging method since malfunction of the Cre/lox system or frame-shift mutations caused by mis-integration of yEGFP would result in loss of function and therefore loss of viability. Subcellular localization of the genomic yEGFP-Ydl193w fusion protein was compared to an episomal N-terminal GFP fusion of YDL193w expressed under control of the *MET25^p* promoter (plasmid pPK199; provided by P. Kötter, EUROSCARF).

A G418 sensitive strain heterozygous for yEGFPgN-YDL193w was sporulated and genomic DNA was analyzed by diagnostic PCR with primers YDL193w_upCtrl (5′-acttttg-gatacttagccgc-3′) and YDL193w_downCtrl (5′-ttctgtcttttg-cttttgga-3′). Two spores displayed a 1.2 kbp fragment (theoretical size: 1274 bp), indicative of correct GFP insertion at the 5′-end of YDL193w, and lack of the *kanMX4* marker (data not shown). Furthermore, correct yEGFP insertion was confirmed by sequencing of the PCR products. In order to test functionality of the tagged protein, spores of a tetrad of the heterozygous diploid yEGFPgN-YDL193w/YDL193w strain were grown in YPD at 30°C overnight and serial dilutions (10^0, 10^{-2}, 10^{-4}, 10^{-6}) were spotted onto YPD plates. All four spores were viable and the growth phenotypes at various temperatures were indistinguishable from wild type (data not shown), suggesting that the promoter manipulation and insertion of additional 53 bp did not grossly alter expression of the YDL193w gene. Furthermore, these data suggest that the N-terminal GFP fusion of the essential gene product of YDL193w is fully functional. To obtain strains that lacked plasmid pSH47 (harboring the URA3 marker), cells were maintained overnight on medium containing 5-fluoro orotic acid (5-FOA).

The fatty acid activation protein Faa2p catalyzes the activation of fatty acids to their acyl-CoA derivatives with a preference for medium chain fatty acids [12]. The non-essential Faa2 protein is localized at the matrix side of the peroxisomal membrane. Import into the peroxisome appears to be dependent on the C-terminal amino acid sequence -EKL which is highly similar to the consensus PTS1 peroxisomal targeting sequence, -SKL. C-terminal Faa2-GFP fusion proteins, either plasmid-borne or expressed from the chromosomal locus, mis-localized to the cytosol due to masking of the carboxy-terminal PTS1 targeting sequence ([13] and data not shown). Transcription of *FAA2* is regulated by the transcription factors Oaf1p and Pip2p (Oaf2p). The gene is repressed during growth on glucose but is highly induced in the presence of oleate [14,15]. Therefore, *FAA2* represented another suitable test model for the N-terminal GFP-tagging technique.

The haploid yEGFPgN-*FAA2* strain, BPY62, was shown to have the correct genomic GFP insert by control PCR with primers FAA2_upCtrl (5′-atttttcagaacatcctccg-3′) and FAA2_downCtrl (5′-gcaaccgcctgtttctt-3′). Additionally, correct in-frame insertion of the yEGFP construct in strain BPY62 was confirmed by sequencing of the PCR product. In order to verify unaltered expression of the tagged *FAA2* gene, strains BPY62 (yEGFPgN-*FAA2*), BPY37 (wild type) and the control mutant strain YAJ8D which is unable to induce peroxisomes and utilize oleate as the sole carbon source [16] were grown overnight in YPD medium. Serial dilutions (10^0, 10^{-2}, 10^{-4}, 10^{-6}) were spotted onto YPD plates and plates containing 0.1% oleate as the sole carbon source. Growth was scored after 3 days of incubation at 15, 30 and 37°C and was indistinguishable between BPY62 and BPY37 whereas YAJ8D, as the negative control, was unable to grow on oleate containing medium. These results suggest full functionality of the yEGFPgN-*FAA2* fusion construct.

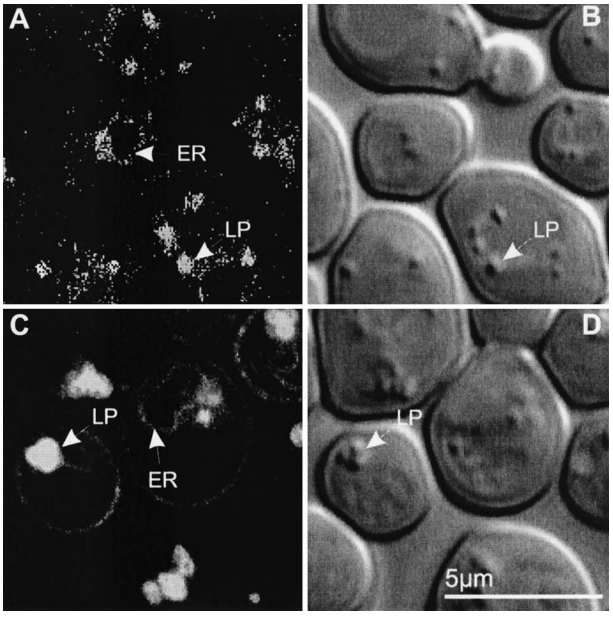

Fig. 2. Microscopic inspection of cells harboring the chromosomal N-terminal yEGFPgN-YDL193w fusion, in comparison to the episomal construct. A,C: GFP fluorescence; B,D: DIC transmission images. A,B: GFP fluorescence of strain BPY46 (homozygous diploid for yEGFPgN-YDL193w) grown to early stationary phase in YPD medium. LP, lipid particles; ER, endoplasmic reticulum. C,D: GFP fluorescence of strain FY1681{pPK199} harboring yEGFP(Nfus)-YDL193w on an episomal plasmid under control of the *MET25^p* promoter. Cells were grown to early stationary phase in glucose minimal medium, containing 0.5 mM methionine for partial derepression of the *MET25^p* promoter. LP, lipid particles; ER, endoplasmic reticulum.

3.3. GFP microscopy

In order to analyze subcellular localization patterns, strain BPY46 was grown in YPD to early stationary phase and prepared for fluorescence microscopy as described in Section 2. The diploid strain FY1681{pPK199} harboring an episomal GFP fusion of YDL193w under control of the *MET25^p* promoter was grown to the same growth phase in minimal medium containing a low methionine concentration in order to achieve partially derepressed levels of gene expression of GFP(Nfus)-YDL193w from the plasmid [17].

GFP fluorescence could be detected in the endoplasmic reticulum and in lipid particles of strain BPY46. The fluorescence intensity was low compared to the plasmid-borne construct, and presumably represents the low endogenous expression level of the YDL193w gene. The localization pattern was indistinguishable for strains BPY46 and FY1681{pPK199} (Fig. 2), however, plasmid-borne GFP fluorescence was rather heterogeneous, and numerous cells lacked any fluorescence. Furthermore, it appeared that overexpression of GFP-Ydl193wp from the plasmid resulted in a clustering of lipid particles that was not observed in strain BPY46, or in wild type by transmission microscopy. Local-

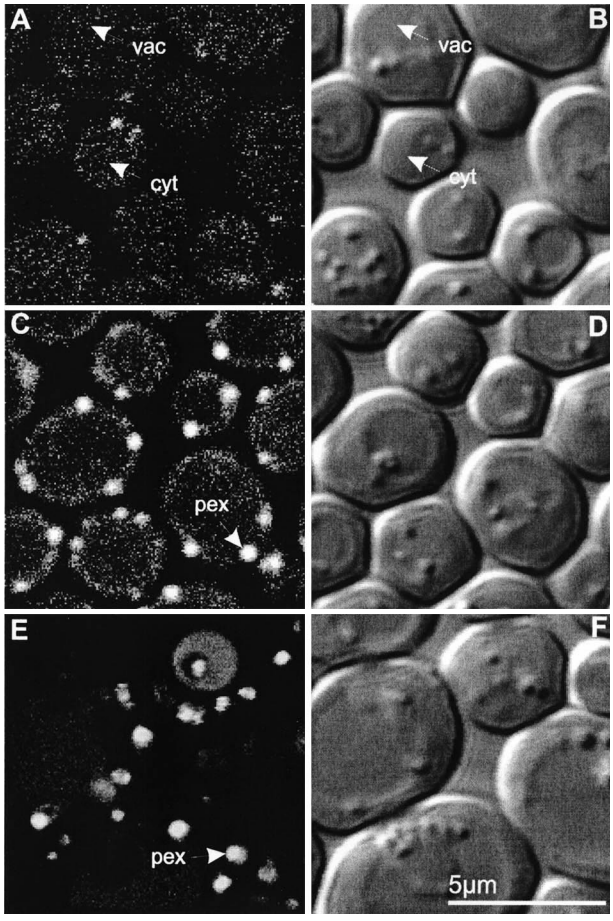

Fig. 3. Microscopic inspection of cells harboring the chromosomal N-terminal yEGFPgN-*FAA2* fusion under non-inducing and inducing growth conditions, in comparison to the episomal construct. A,C,E: GFP fluorescence; B,D,F: DIC transmission images. A,B: GFP fluorescence of strain BPY62 (yEGFPgN-*FAA2*), under non-inducing growth conditions in YPD. Fluorescence appears at background level, in the cytosol (cyt) and the vacuole (vac). C,D: GFP fluorescence of strain BPY62 (yEGFPgN-*FAA2*) under inducing growth conditions in medium containing oleate. GFP-Faa2p localizes to punctate structures that resemble peroxisomes (pex). E,F: GFP fluorescence of strain BPY63 (*MET25*^{p}-yEGFP(Nfus)-*FAA2*) expressing the *GFP-FAA2* fusion from an episomal plasmid, during early stationary phase of growth in minimal medium, containing oleate as the sole carbon source and 0.5 mM methionine for partial derepression of the *MET25*^{p} promoter. Plasmid-borne GFP-Faa2p localizes to peroxisomes (pex) under inducing conditions.

ization of GFP-Ydl193wp to lipid particles is consistent with previous reports [11]; a dual localization to both lipid particles and the endoplasmic reticulum has previously been reported for several proteins involved in ergosterol biosynthesis [18] and once more reflects a relationship between these two organelles. Localization to lipid particles suggests a potential role for Ydl193wp in lipid metabolism.

yEGFPgN-Faa2p fluorescence, under non-inducing conditions, i.e. in the presence of glucose in the growth medium, was barely detectable and mostly resided in the cytosol or vacuole (Fig. 3A). Upon induction of peroxisome formation with oleic acid as the sole carbon source, the fusion protein became strongly induced and localized to punctate structures, resembling peroxisomes (Fig. 3B). An N-terminal GFP(Nfus)-Faa2 fusion protein expressed from a plasmid under control of the *MET25*^{p} promoter was observed exclusively in peroxisomes of cells grown on oleate (Fig. 3C). In these cells grown on glucose and in the absence of methionine (derepression of the *MET25*^{p} promoter), most of the GFP fusion protein localized to the cytosol and the vacuole, due to the lack of peroxisomes (data not shown). This demonstrates that expression from episomal plasmids under heterologous promoter control may give misleading results about protein localization. Correct localization to peroxisomes and oleate-regulated expression patterns of cells harboring the chromosomally integrated N-terminal GFPgN-*FAA2* fusion further confirm the functionality and feasibility of the N-terminal yEGFP-tagging technique in yeast.

In this article we describe a novel and efficient method for N-terminal tagging of *S. cerevisiae* proteins with GFP. The method combines the advantages of chromosomal tagging with GFP, namely stable integration, expression that is under control of the endogenous promoter, homogeneous staining of the cell population and independence of selective media for plasmid maintenance, with the ease of a PCR-mediated procedure lacking any cloning steps. Although this method involves one additional transformation with the Cre recombinase plasmid, compared to the PCR-based method for C-terminal GFP fusions [1–4], it may prove useful under circumstances when C-terminal tagging is not possible due to specific protein targeting or modification sites, or loss of function due to the C-terminal tag.

Using two test constructs, namely the essential gene YDL193w as well as the oleate-inducible gene *FAA2*, we found that insertion of additional 53 bp upstream of the GFP translation start site, as a consequence of the marker excision procedure, did not affect viability of the transformed cells or the expression pattern of the respective genes. Although subtle changes in expression levels that may also be due to the GFP tag attachment cannot be ruled out, the method described here represents a suitable and simple experimental alternative to episomal N-terminal GFP-tagging in *S. cerevisiae*.

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