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Integrative, multifunctional plasmids for hypha-specific or constitutive expression of green fluorescent protein in *Candida albicans*

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The authors have engineered plasmid constructs for developmental and constitutive expression of yeast-enhanced green fluorescent protein (yEGFP3) in *Candida albicans*. The promoter for the hyphae-specific gene Hyphal Wall Protein 1 (*HWP1*) conferred developmental expression of yEGFP3 in germ tubes and hyphae but not in yeasts or pseudohyphae when targeted to the *ENO1* (enolase) locus in single copy. The pHWP1GFP3 construct allows for the easy visualization of *HWP1* promoter activity in individual cells expressing true hyphae without having to prepare RNA for analysis. Constitutive expression of yEGFP was seen in all cell morphologies when the *HWP1* promoter was replaced with the *ENO1* promoter region. The use of the plasmids for expression of genes other than yEGFP3 was examined by substituting the putative *C. albicans BCY1* (*SRA1*) gene, a component of the cAMP signalling pathway involved in yeast to hyphae transitions, for yEGFP3. Strains overexpressing *BCY1* from the *ENO1* promoter were inhibited in germ tube formation and filamentation in both liquid and solid media, a phenotype consistent with keeping protein kinase A in its inactive form by association with Bcy1p. The plasmids are suitable for studies of germ tube induction or assessing germ tube formation by measuring yEGFP3 expression, for inducible expression of genes concomitant with germ tube formation by the *HWP1* promoter, for constitutive expression of genes by the *ENO1* promoter, and for expressing yEGFP3 using a promoter of choice.

INTRODUCTION

A virulence characteristic of the human fungal pathogen *Candida albicans* is its ability to grow in various morphological forms. The conversion between yeasts, elongated yeasts (pseudohyphae) and filamentous forms in response to environmental cues is accompanied by differential expression of several genes regulated at the transcriptional level (reviewed by Calderone & Fonzi, 2001; Ernst, 2000; Liu, 2001). The expression of the Hyphal Wall Protein 1 gene (*HWP1*) is tightly controlled at the transcriptional level; *HWP1* mRNA and protein are absent in yeasts and pseudohyphae (Staab *et al.*, 1996, and unpublished results). The transcriptional regulation of *HWP1* and other genes regulated by the bud–hypha transition, such as *HYR1*, *RBT1* and *ECE1* (Braun & Johnson, 2000), suggests that *cis* elements in the promoter regions respond to signals that control morphology. Several signalling pathways and transcriptional factors have been described that promote the formation of germ tubes and hyphae but common promoter elements in hypha-specific genes proven to be essential for expression have yet to be uncovered. Functional analysis of the *HWP1* promoter will provide insights into common regulatory elements of other genes controlled by the bud–hypha transition. With this aim, we constructed a reporter plasmid that would permit easy visualization of *HWP1* promoter activity by using yeast-enhanced green fluorescent protein (Cormack *et al.*, 1997) as a readout. Green fluorescent protein (GFP) has been widely used in cell biology to monitor gene expression and cellular localization of proteins (Chalfie *et al.*, 1994; Gerami-Nejad *et al.*, 2001; Niedenthal *et al.*, 1996). We engineered the reporter plasmid with the selectable marker gene *URA3* within the targeting DNA sequences to direct insertion at the *ENO1* locus of a Ura$^-$ strain. As a counterpart to the developmentally expressed yEGFP3 construct, we replaced the *HWP1* promoter with the *ENO1* promoter to express relatively high amounts of GFP in a constitutive manner.

To demonstrate the use of the plasmids in expressing genes other than GFP, we replaced the yEGFP3 gene with the putative *C. albicans BCY1* (*SRA1*, regulatory subunit of cAMP-dependent protein kinase A, PKA), a member of the cAMP signalling pathway. The cAMP signalling pathway is known to be involved in germ tube formation (Bahn & Sundstrom, 2001; Chattaway *et al.*, 1981; Niimi, 1996; Niimi *et al.*, 1980; Zelada *et al.*, 1996), and perturbations of cAMP levels or PKA activity can induce or inhibit germ

Abbreviations: GFP, green fluorescent protein; PKA, protein kinase A.

tube formation (Bahn & Sundstrom, 2001; Castilla *et al.*, 1998; Chattaway *et al.*, 1981). Signals that increase cAMP levels or addition of exogenous cAMP or dibutyryl cAMP activate the cAMP pathway and promote germ tube formation (Bahn & Sundstrom, 2001; Castilla *et al.*, 1998; Chattaway *et al.*, 1981). If, on the other hand, the release of active subunits of PKA is blocked, the cAMP signalling pathway is deactivated, and germ tube induction is suppressed. Overexpression of the regulatory subunit of PKA should prevent the release of active PKA subunits and abrogate the activation of genes involved in germ tube formation. Thus, the expected phenotype of strains overproducing Bcy1p is a reduction in germ tube formation.

The plasmids described here offer several uses as tools for molecular genetic research in *C. albicans*. The developmental expression of GFP by the *HWP1* promoter was maintained even when the construct was integrated ectopically at the *ENO1* locus. Constitutive expression of yEGFP3 from the *ENO1* promoter permitted visualization of GFP in all cell types, and served as a control for a non-developmentally regulated promoter. Lastly, the versatility of the constructs was tested by substituting yEGFP3 for *BCY1*, a member of the cAMP signalling pathway, to determine the effect of mis-expression or overexpression of *BCY1* on filamentation.

METHODS

Construction of an *HWP1* promoter expression plasmid. All enzymes were from Gibco-BRL Life Technologies or Promega Biotech and were used according to standard techniques (Sambrook *et al.*, 1989). Polymerase chain reactions (PCR) were performed to amplify components of the expression vector. TaqPlus DNA polymerase (Gibco-BRL) was used, unless otherwise specified, according to the manufacturer's recommendations. The −1410 to +67 region upstream of the *HWP1* open reading frame was amplified with oligonucleotides 5′GGCCCGGGATCTTTCTTTTTCATTTCCC3′ and 5′GGAAGCTTATTGACGAAACTAAAAGCA3′ engineered with *Sma*I and *Hin*dIII sites, respectively (underlined nucleotides) using a genomic *C. albicans* DNA plasmid clone isolated from an SC5314 (Gillum *et al.*, 1984) genomic library (Birse *et al.*, 1993) harbouring a *Bgl*II fragment encompassing nucleotides −1410 to +120. The 1·46 kbp PCR product was ligated to the *Hin*cII and *Hin*dIII sites of pBluescript SK$^-$ (Stratagene) to create pBS5′. The codon-optimized *Aequorea victoria* GFP gene, yEGFP3, was released from pYGFP3 (Cormack *et al.*, 1997) by digestion with *Hin*dIII and *Pst*I, and the gene fragment was cloned downstream of the *HWP1* promoter between the *Hin*dIII and *Pst*I sites of pBS5′ to generate pBS147GFP. The untranslated 3′ region of *HWP1* was amplified with oligonucleotides 5′GGTATTGCTGCATTCTTGATCTAATTC3′ and 5′GGACAGAGCTCACATTTTCTACCAATTAAACCACTGAATAAGCATAGAAC3′. A *C. albicans* DNA plasmid clone having a 3 kbp *Bam*HI fragment with C-terminal and downstream *HWP1* sequences was used as template (pGB23; Staab & Sundstrom, 1998) with *Pfu* DNA polymerase (Stratagene) to generate a blunt-ended DNA amplification product. The right oligonucleotide was chosen to amplify the 3′ region of *HWP1* ending at the unique *Sac*I site (double-underlined nucleotides) and across a *Hin*dIII site that was eliminated by changing a T to an A (nucleotide in bold). The 342 bp PCR product was digested with *Sac*I and ligated to the *Sma*I and *Sac*I sites of p147GFP to create p147GFP3′. A *URA3*-disrupted *ENO1* DNA fragment was used as a selectable marker and targeting sequences for integration at the chromosomal *ENO1* locus. The entire *ENO1* ORF was amplified with oligonucleotides engineered with *Xba*I (5′ primer, underlined) and *Xho*I sites (3′ primer, double underlined): 5′GGTCTAGACAGGAATATTACAACAATGTCTTACGC3′ and 5′GGCTCGAGCAGAGGCAAACTTACAATTGAGAAGCC3′. The amplified product was cloned into pBluescript SK$^-$ (Stratagene) at the *Xba*I and *Xho*I sites to generate pENO1. The 5′ *Hin*dIII site and the unique *Cla*I in *ENO1* were mutagenized by site-directed mutagenesis (Promega GeneEditor) to eliminate the *Hin*dIII site and to prevent methylation adjacent to the *Cla*I site (*Cla*I is site-specific methylation sensitive at the *dam* site; McClelland *et al.*, 1994). The 125 bp between the two remaining *Hin*dIII sites in *ENO1* was replaced with the *URA3* ORF found in the 1·44 kbp *Rsa*I fragment (Kelly *et al.*, 1988) in p5921 (Fonzi & Irwin, 1993) after creating blunt ends at the *Hin*dIII sites with Klenow fragment DNA polymerase (Sambrook *et al.*, 1989). The *eno1*::*URA3* construct was subsequently amplified by PCR with oligonucleotides having *Kpn*I sites at the ends (underlined): 5′GGGGTACCATGTCTTACGCCACTAAAATCCAC3′ and 5′GGGGTACCCCAGCGTAGATAGCTTCAGAACCT3′. The 2·6 kbp PCR product was digested with *Kpn*I and cloned into the unique *Kpn*I site of p147GFP3′ to generate pHWP1GFP3. Each construct was analysed for proper cloning at the DNA level by sequencing across ligation junctions (automated cycle sequencing, ABI Prism, model 377 and 373, Perkin-Elmer). A promoterless construct was created by digesting pHWP1GFP3 with *Xho*I and *Hin*dIII, purifying the vector away from the 1·47 kbp 5′ *HWP1* fragment, and incubating with Klenow fragment DNA polymerase to produce blunt DNA ends (Sambrook *et al.*, 1989). Self-ligation of the blunt-ended vector produced p0GFP3.

Substitution of the *HWP1* promoter in pHWP1GFP3 with the *ENO1* promoter. The constitutive expression of yEGFP3 was achieved by replacing the *HWP1*p with the *ENO1*p region. The contig encoding *ENO1* was identified (ORF6.6269 on Contig 2451) at Stanford's *Candida albicans* Sequencing Project Assembly 6 (see URL below), and the DNA sequence information used to amplify the promoter region from −900 to +36 with oligonucleotides engineered with *Xho*I (underlined) and *Hin*dIII sites (double underlined), respectively: 5′CCCCCTCGAGTTTTGAAAGGTCTGTCATATTTCTAT3′ and 5′CCCCAAGCTTTGTTGTAATATTCCTGAATTATCAATTGATC3′. Wild-type genomic DNA from SC5314 served as template. The 955 bp PCR product was digested with *Xho*I and *Hin*dIII and cloned into pBluescript SK$^-$ (Stratagene) at the *Xho*I and *Hin*dIII sites. Once the *ENO1*p region was verified by DNA sequencing, the insert was excised with *Xho*I and *Hin*dIII and used to replace the *HWP1*p in pHWP1GFP3 cloned between the *Xho*I and *Hin*dIII sites. The new recombinant plasmid was named pENO1GFP3.

Substitution of yEGFP3 with *BCY1* (*SRA1*). The amino acid sequence of *Saccharomyces cerevisiae* Bcy1p obtained at the *Saccharomyces* Genome Database (http://www.yeastgenome.org/) was used to search the *C. albicans* genome at Stanford's *Candida albicans* Sequencing Project Assembly 6 (http://sequence-www.stanford.edu/group/candida/index.html) for a homologous gene product. ORF 6.2117, named *SRA1*, coded for a 459 amino acid protein with 47 % identity to *S. cerevisiae* Bcy1p (Sra1p). Because the preferred gene name at the *Saccharomyces* Genome Database is *BCY1*, the *C. albicans* putative homologue was also designated *BCY1* (*CaBCY1*). The entire *C. albicans BCY1* ORF was generated by PCR using *Pfu* polymerase, SC5314 genomic DNA as template and two oligonucleotides, 5′CCCAAGCTTATGTCTAATCCTCAACAGCA3′ and 5′GGGCTGCAGTTAATGACCAGCAGTTGGGT3′, engineered with *Hin*dIII (underlined) and *Pst*I (double-underlined) sites. The yEGFP3 gene fragment in pENO1GFP3 was replaced with the

1·37 kbp *BCY1* PCR product digested with *Hin*dIII and *Pst*I, to generate pENO1BCY1. The authenticity of *BCY1* was confirmed by automated cycle sequencing as above.

Transformation of *C. albicans* with GFP and *BCY1* plasmids, and verification of plasmid integration at *ENO1*. Plasmid constructs were targeted to the chromosomal *ENO1* locus by digesting the plasmids at the unique *Cla*I site prior to transformation of the *ura3 C. albicans* strain CAI4 (Fonzi & Irwin, 1993). Strain CAI4 was transformed with 5 μg linearized DNA using the protoplasting method (Kurtz *et al.*, 1986), and stable transformants were streaked for isolation onto yeast nitrogen base plates (YNB, 50 mM glucose). Single-copy integrations of the plasmid constructs were verified by Southern blotting of genomic DNA digested with *Bgl*II probed with cENO1 (Postlethwait & Sundstrom, 1995) directly labelled with horseradish peroxidase (Amersham Pharmacia) and developed with chemiluminescence reagents (Pierce).

Induction of GFP expression in *C. albicans* transformants. Yeast strains grown to stationary phase on YNB plates or in liquid medium at 30 °C were used as inoculum for 30 °C yeast peptone dextrose [glucose] (YPD), 37 °C YPD plus 10 % bovine calf serum (Sigma) (Braun & Johnson, 2000), or 37 °C Medium 199 (M199, Life Technologies) as before (Bahn & Sundstrom, 2001; Staab *et al.*, 1996). The cells were allowed to germinate at 37 °C or grow as budding yeasts at 30 °C for 2–3 h before microscopic examination by epifluorescence using a fluorescein isothiocyanate (470–490 nm excitation/515–550 nm emission) cube. Expression of yEGFP3 regulated by the *HWP1* promoter was also assessed by growing yeasts to exponential phase in modified Lee's media (Brummel & Soll, 1982; Staab *et al.*, 1996; Sundstrom & Aliaga, 1994; Sundstrom *et al.*, 1990). Cells were photographed at 400 × magnification with an Olympus BX60 microscope fitted with a MagnaFire S99806 camera. Images were manipulated with Adobe PhotoShop 5.0.

yEGFP3 expression was also induced in agar-containing media (Lo *et al.*, 1997). Stationary-phase yeasts grown in YNB were mixed (100 cells in 25 ml) with liquefied 2 % agar containing 4 % bovine calf serum and poured into plates. The hardened plates were incubated at 37 °C for up to 7 days. Colonies were photographed under epifluorescence at 20 × magnification as above.

Analysis of filamentation in solid and liquid media. Strains transformed with pHWP1GFP3 (HGFP3), pENO1GFP3 (EGFP3) and pENO1BCY1 (EBCY1) were induced to form agar-embedded filamentous colonies in Spider medium (Liu *et al.*, 1994) and 2 % agar with 4 % bovine calf serum plates as described above. Stationary-phase cells grown in YNB were mixed (200 cells in 25 ml) with the agar-containing media and poured into Petri dishes. The hardened plates were incubated at 37 °C for 7–10 days. Colonies were photographed at 1 × magnification with a stereoscope (Olympus SZX12) fitted with a MagnaFire S99806 camera. Germ tube formation was also assessed by growth in liquid M199 at 37 °C as before (Bahn & Sundstrom, 2001). Images were manipulated with Adobe PhotoShop 5.0.

Northern blot analysis. Total RNA was prepared (Schmitt *et al.*, 1990) from CAI4 transformed with pENO1GFP3 (EGFP3, control strain) and pENO1BCY1 (EBCY1). RNA was isolated from exponential-phase cells growing in modified Lee's media at pH 4·5 at 25 °C (yeasts) and pH 6·8 at 37 °C (germ tubes), and analysed in standard formaldehyde gels (10 μg RNA per lane) followed by blotting onto nitrocellulose membranes as before (Staab *et al.*, 1996). The membranes were probed with ^{32}P-labelled *BCY1* ORF used to construct pENO1BCY1 (see above), and with a probe for 18S rRNA (Bahn & Sundstrom, 2001).

RESULTS AND DISCUSSION

Plasmid constructs integrated at the *ENO1* locus retain promoter regulation

Our goals were to construct plasmids for the study of the hypha-specific gene promoter, *HWP1*, which would permit easy visualization of promoter activity combined with simple replacement of promoter and reporter gene fragments. The availability of a codon-optimized yEGFP3 gene fragment (Cormack *et al.*, 1997) was ideal in that it allowed for easy visualization of gene expression. The expression plasmid included 1·47 kbp of upstream *HWP1* DNA and 352 bp of 3′ untranslated *HWP1* (Staab & Sundstrom, 1998) (Fig. 1). A *URA3*-disrupted *ENO1* fragment was a convenient way to include a selectable marker within the targeting sequences. Previous studies in our laboratory have shown that disruption of one *ENO1* homologue is not detrimental to cell growth on glucose or pyruvate, and does not affect virulence as measured in the murine systemic

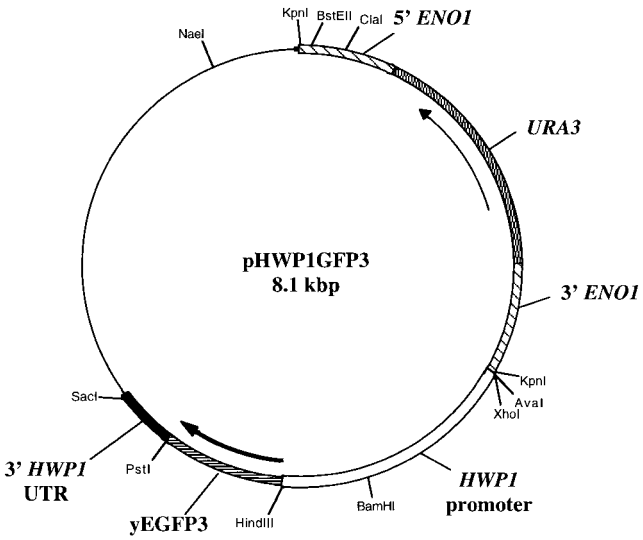

Fig. 1. Schematic map of the HWP1GFP3 integration plasmid. The *HWP1* promoter (open region) controls expression of yEGFP3 (closely hatched segment), followed by the *HWP1* 3′ untranslated region (black segment). The targeting/selection sequences, *eno1*::*URA3*, are shown as widely hatched segments (*ENO1* sequences) interrupted by a closely double-hatched region (*URA3*). The thick and thin arrows represent the direction of transcription of yEGFP3 and *URA3*, respectively. The plasmid was integrated at the *ENO1* locus after digestion with *Cla*I prior to transforming the Ura$^-$ strain, CAI4. Digestion with *Bst*EII also directs integration of the construct to the *ENO1* locus (data not shown). To create pENO1GFP3, the *HWP1* promoter was replaced between the unique *Xho*I and *Hin*dIII sites with 0·9 kbp 5′ *ENO1* sequences. The expression plasmids were constructed in the vector pBluescript SK$^-$ (Stratagene) (thin black arc). Single restriction enzyme sites and gene segment descriptions are shown on the periphery of the plasmid.

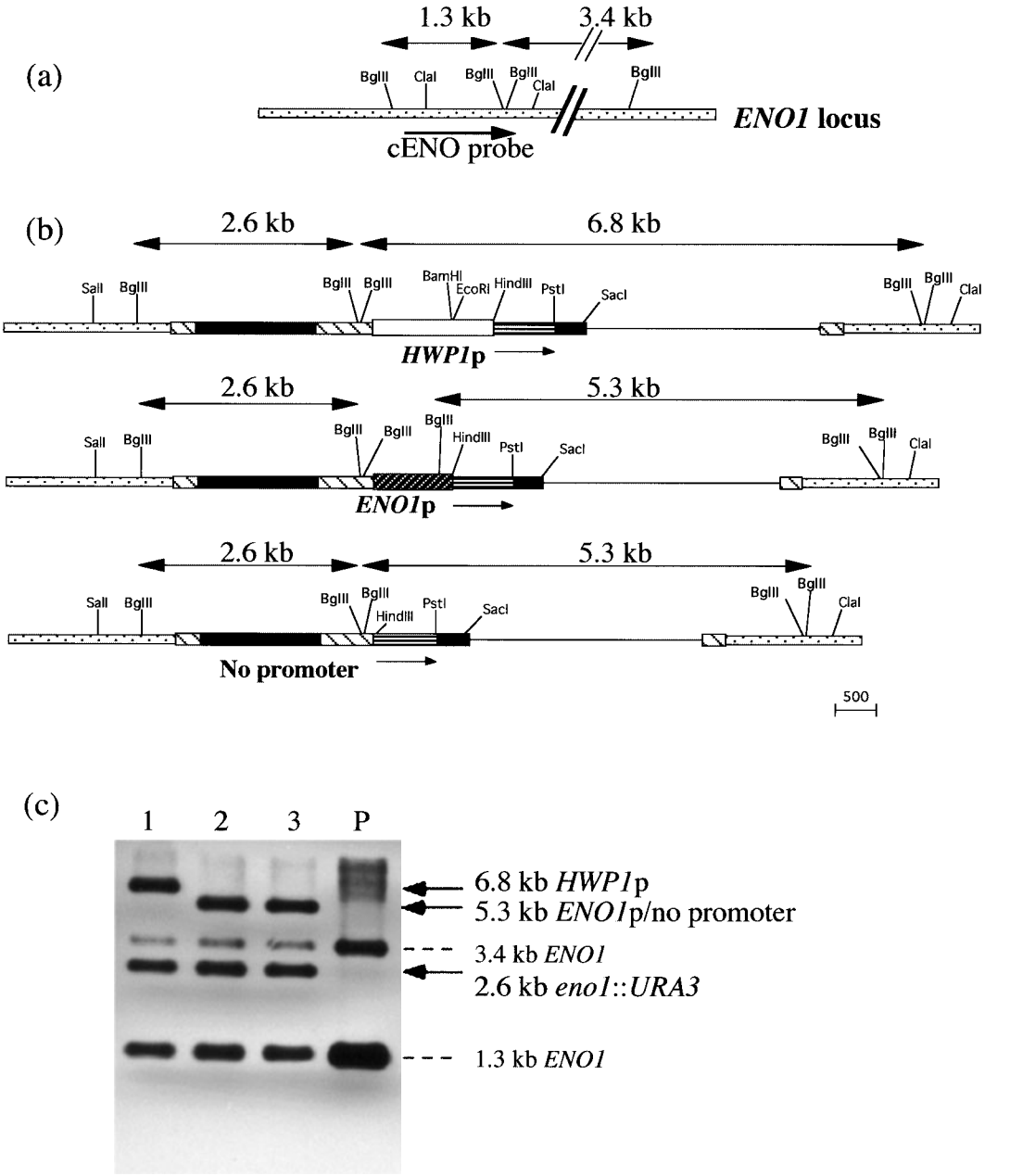

Fig. 2. Integration of the GFP expression plasmids into the chromosome of *C. albicans* at the *ENO1* locus. (a) Restriction map of the *ENO1* locus with the predicted Southern blot hybridization fragments shown above as double-headed arrows. *Bgl*II digestion of genomic DNA generates two fragments, of 1·3 and 3·4 kbp, which hybridize to an *ENO1* probe comprising the entire *ENO1* ORF (cENO, black arrow below the *ENO1* locus; the probe has limited homology to the wild-type 3·4 kbp fragment). (b) Diagrams of the *HWP1*, *ENO1* and no promoter constructs (top to bottom, respectively) integrated at the *ENO1* locus. Double-headed arrows above the gene maps indicate the sizes of the *Bgl*II fragments hybridized by the cENO probe. Thin black arrows below the gene maps show the direction of transcription of yEGFP3 (represented by rectangles filled with thin parallel lines). Restriction sites are shown above the gene maps. Scale bar, 500 bp. (c) Southern blot analysis of *Bgl*II-digested genomic DNA from strains transformed with the yEGFP3 plasmids probed with cENO (arrow below *ENO1* locus in a). Lane 1, strain HGFP3 (CAI4 transformed with pHWP1GFP3) contains the expected two new fragments of 6·8 and 2·6 kbp (arrows at right) in addition to the *ENO1* gene fragments from intact homologues (dashed lines at right, see lane P). Lane 2, strain EGFP3 (CAI4 transformed with pENO1GFP3) contains the expected two new fragments of 5·3 and 2·6 kbp (arrows at right) in addition to the two fragments from intact homologues (dashed lines at right). The cENO probe does not recognize the central *Bgl*II fragment within the *ENO1* promoter (see b). Lane 3, strain 0GFP3 (CAI4 transformed with the promoterless plasmid) contains two new fragments of 5·3 and 2·6 kbp (arrows at right) in addition to the two fragments from the intact *ENO1* homologues (dashed lines at right). Lane P, parental strain CAI4.

candidiasis model (Postlethwait & Sundstrom, 1995; Sundstrom *et al.*, 2002). Initially we used the *eno1*::*URA3* fragment previously generated in our laboratory (Postlethwait & Sundstrom, 1995), but we noticed a tendency for multiple tandem copies of the plasmid to integrate into the chromosome (data not shown), an observation reported by others using a different chromosomal locus (Srikantha *et al.*, 1995). Multiple tandem integrations of transforming DNA have also been well documented in the yeast *Pichia pastoris* (Clare *et al.*, 1991). In an attempt to minimize plasmid copy number and etopic integrations, a longer region of homology to the 5′ region of *ENO1* was incorporated into a new *eno1*::*URA3* fragment (see Methods). Fewer plasmid integrations occurred with the new construct, and the *C. albicans* transformation efficiency increased by 10–100-fold (data not shown). Subsequently, two derivative plasmids were constructed by substituting the *HWP1*p for the constitutive *ENO1*p to generate pENO1GFP3, and by deleting promoter sequences to create p0GFP3 (Fig. 2b). Southern blot analysis of Ura$^+$ transformants confirmed site-specific integration of the plasmid constructs at the *ENO1* locus (Fig. 2c). PhosphorImager analysis of genomic DNA probed for *ACT1* (actin) and *ENO1* sequences (Postlethwait & Sundstrom, 1995) revealed single plasmid integrations at two or three out of the four *ENO1* homologues (data not shown). The low copy number is similar to that conferred by *CEN* episomal vectors in *S. cerevisiae* which are maintained in one to two copies per cell (Bloom *et al.*, 1983). Analyses of multiple independent transformants of each construct did not reveal growth or germination defects (data not shown).

A critical factor validating the *HWP1*p construct was maintaining developmental regulation of the *HWP1*p outside of the *HWP1* locus. Epigenetic regulation of *HWP1* could prevent use of the construct if developmental yEGFP3 expression was lost at the *ENO1* locus. Analysis of yEGFP3 expression in yeasts, pseudohyphae and hyphae by growing cells in all four modified Lee's media (Brummel & Soll, 1982; Staab *et al.*, 1996) confirmed the developmental regulation of the reporter gene (Fig. 3a). GFP was only observed in true hyphae, paralleling the expression pattern of *HWP1* (Staab *et al.*, 1996); pseudohyphae (arrows in Fig. 3a) and yeasts were negative for green fluorescence. Thus, the construct contained all the necessary *cis* elements for developmental expression, and ectopic placement of the *HWP1* promoter did not change its regulation.

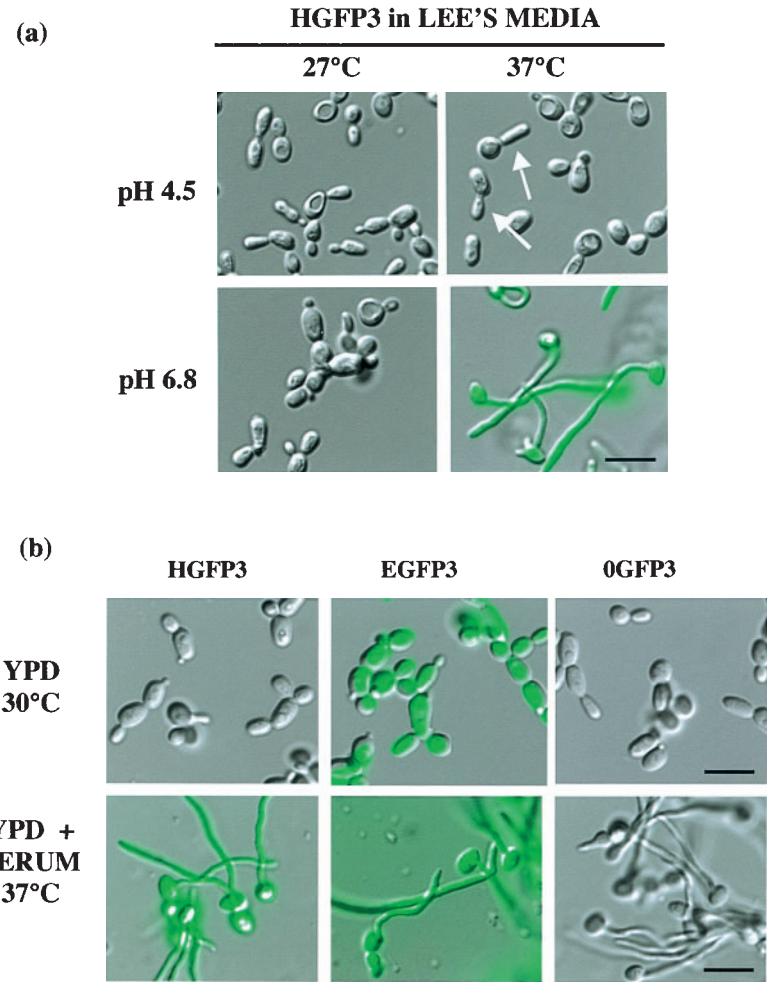

Fig. 3. Expression of GFP in *C. albicans*. (a) Strain HGFP3 expresses GFP in a hyphae-specific manner, paralleling the expression pattern of *HWP1*. Mid-exponential-phase cells were grown in all four Lee's media for 3 h prior to microscopic examination. White arrows point to GFP-negative cells forming pseudohyphae in Lee's pH 4·5 at 37 °C. Scale bar, 10 μm. (b) Strains expressing GFP controlled by the *HWP1*, *ENO1* and no promoter constructs. Stationary-phase yeast cells were diluted into fresh YPD or YPD plus 5% bovine calf serum, and grown at 30 °C or 37 °C, respectively, for 3 h prior to microscopic examination. The *ENO1* promoter (EGFP3) drives constitutive expression of GFP regardless of cell type (middle column), while the promoterless construct is negative for GFP (right column). The *HWP1* promoter (left column) maintained developmental regulation of GFP. Scale bars, 10 μm.

Constitutive expression of yEGFP3 was achieved when the *HWP1*p was replaced with the *ENO1*p (Fig. 3b). All cell types of EGFP3 grown in the four modified Lee's media were also brightly fluorescent (data not shown). Cells transformed with the promoterless construct were negative for GFP as expected (Fig. 3b).

Filamentation and yEGFP3 expression were examined in solid serum plates (Fig. 4). Embedded colonies expressed GFP when either the *HWP1* or the *ENO1* promoter controlled expression of yEGFP3. Closer examination of budding branches near the ends of HGFP3 filaments revealed GFP-negative buds and pseudohyphae (Fig. 4, arrows) suggesting that developmental regulation of yEGFP3 expression was maintained by the *HWP1* promoter in solid medium. GFP was observed in all cell types in strain EGFP3 as expected.

Constitutive expression of *BCY1* diminishes germ tube formation

The versatility of the constructs was tested by substituting yEGFP3 with *CaBCY1* (*SRA1*), a gene encoding the regulatory subunit of cAMP-dependent PKA, a component of the cAMP signalling pathway. Since the increase in intracellular cAMP levels positively affects germ tube formation (Bahn & Sundstrom, 2001; Castilla *et al.*, 1998; Chattaway *et al.*, 1981), degradation of cAMP should diminish or inhibit filamentation. In *S. cerevisiae*, increasing the expression of *BCY1* shifts the equilibrium of association/dissociation of PKA from subunits towards the associated (inactive) state even in the presence of cAMP (Portela *et al.*, 2001), thus blunting downstream effects of PKA on gene activation. Therefore, if PKA activity is regulated by an analogous mechanism in *C. albicans*, we expected that overexpression of *BCY1* should reduce germ tube formation and

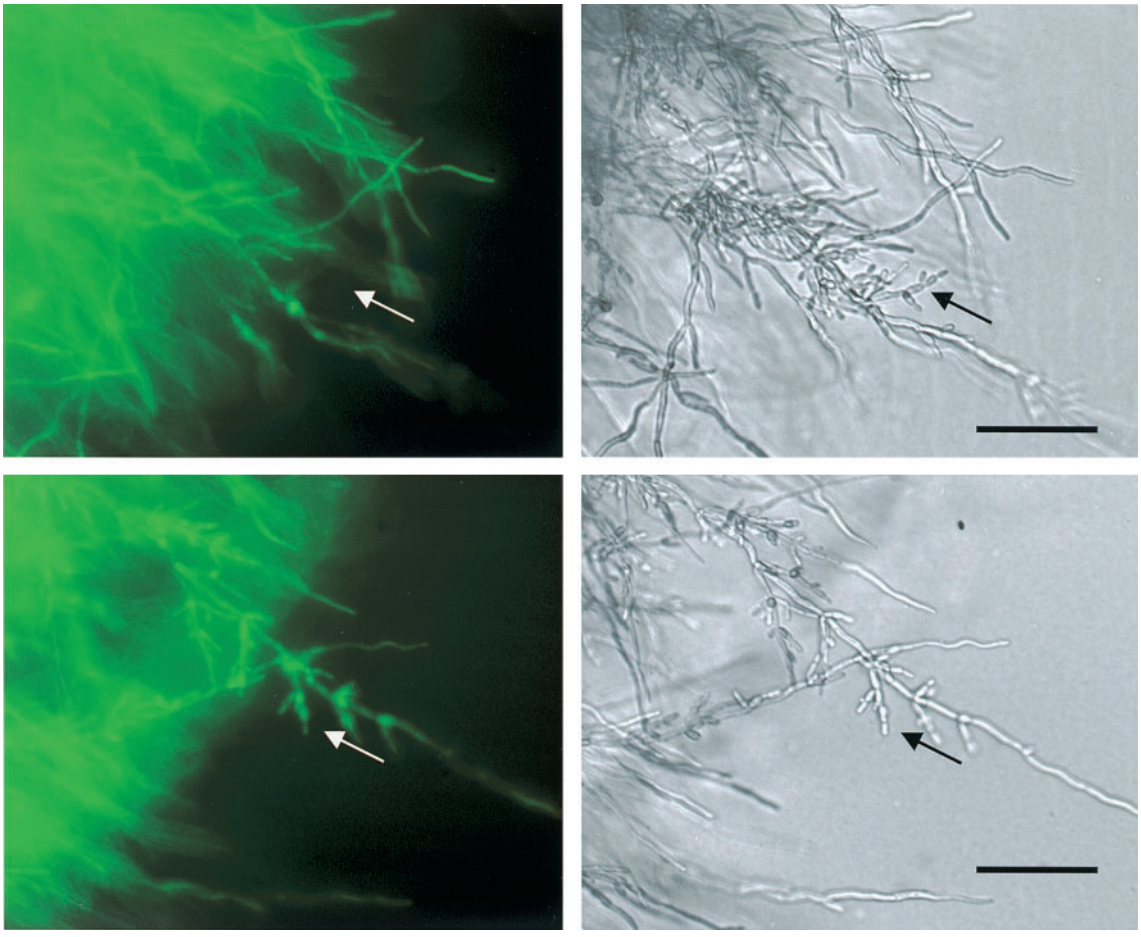

Fig. 4. Expression of yEGFP3 in cells embedded in serum agar medium. Strains transformed with the *HWP1* (HGFP3; top row) or *ENO1* (EGFP3; bottom row) promoter constructs were plated in 4 % bovine serum, 2 % agar plates and incubated at 37 °C for 7–10 days. GFP production and colonial morphologies were observed by fluorescence (left) and light (right) microscopy, respectively. Arrows point to yeasts and elongated yeasts budding laterally from hyphae. Developmental regulation of yEGFP3 expression was maintained in HGFP3 (top row; arrows point to GFP-negative yeasts) while EGFP3 expressed GFP in all cell types (bottom row). Scale bars, 50 μm.

filamentation. Constitutive overexpression of *BCY1* by the *ENO1*p indeed produced strains deficient in filamentation. Strain EBCY1 was deficient in germ tube formation and filamentation in liquid and solid media, respectively (Fig. 5b, c). *BCY1* mRNA was overexpressed in both EBCY1 yeasts and germ tubes relative to the control strain EGFP3 (Fig. 5a), consistent with the morphology-independent expression of the *ENO1* gene (Postlethwait & Sundstrom, 1995; Staab *et al.*, 1996). EBCY1 continued to form elongated yeasts when placed in M199 at 37 °C (Fig. 5b, white arrow) without forming true hyphae (Fig. 5b, black arrow). No change in the doubling times of EBCY1 yeasts relative to EGFP3 or to another control strain, UnoPP-1 (Postlethwait & Sundstrom, 1995), was noted (data not shown), indicating that a continuous abundance of Bcy1p did not affect growth and perhaps indirectly interfere with germ tube formation. The inhibition of true hyphae formation in strain EBCY1 suggests that overexpression of *BCY1* leads to titration of free and active PKA subunits into bound and inactive molecules unable to signal downstream gene targets involved in germ tube formation. The filamentation defect of EBCY1 was also seen in colonies embedded in Spider and serum agar media (Fig. 5c). The morphology of EBCY1 cells at the periphery of the colonies in both solid media was mostly yeasts or very short germ tubes. Constitutive expression of yEGFP3 in the control strain, EGFP3, did not affect normal filamentation in either medium. Copious hyphae (in serum plates) and hyphae with branching yeasts (in Spider plates) were seen at the periphery of embedded colonies of EGFP3, phenotypes that are associated with filamentation-competent strains (Bahn & Sundstrom, 2001; Liu *et al.*, 1994; Lo *et al.*, 1997). The data suggest that induction of germ tube formation or filamentation by different environmental signals in EBCY1 was not enough to surpass the effect of constitutive overexpression of *BCY1* by the *ENO1*p. The results confirmed the expected filamentation-defective phenotype of overexpressing *BCY1* and subsequent inactivation of

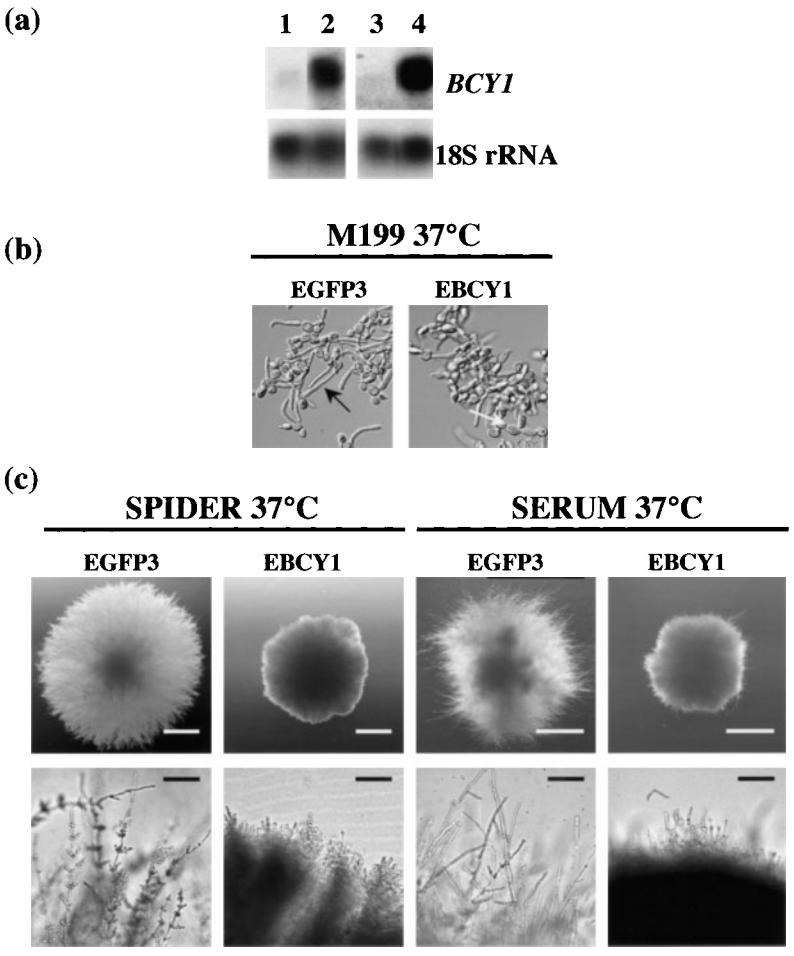

Fig. 5. Overexpression of *BCY1* inhibits germ tube formation and filamentation. (a) Overexpression of *BCY1* by the *ENO1* promoter. Northern blot analysis of *BCY1* mRNA expression driven by the *ENO1* promoter in yeasts (lanes 1 and 2) or germ tubes (lanes 3 and 4). *BCY1* mRNA levels are higher in EBCY1 (lanes 2 and 4) than in the wild-type strain SC5314 (lanes 1 and 3). A probe for 18S RNA (see Methods) served as control for total RNA loaded per lane (bottom row). (b) Inhibition of germ tube formation by overexpression of *BCY1*. Control (EGFP3) and EBCY1 yeasts were induced to form germ tubes in liquid M199 at 37 °C for 3 h. After incubation, the control strain (EGFP3) formed germ tubes (black arrow) whereas EBCY1 grew mostly as yeasts or elongated yeasts (white arrow). (c) Filamentation in solid media. EBCY1 and the control strain (EGFP3) yeast cells embedded in Spider (left two columns) and serum (right two columns) media were grown at 37 °C for several days. EGFP3 produced typical filamentous colonies in both media (top row) while the EBCY1 strain formed more compact colonies lacking filamentous projections. Closer examination of the periphery of the colonies of EGFP3 revealed filamentous cells with branching buds in Spider medium (first column, second row) absent in EBCY1 colonies (second column, second row). Serum medium produced EGFP3 colonies with copious hyphae (third column, second row) not seen at the periphery of EBCY1 colonies (fourth column, second row). White and black scale bars represent 0·5 mm and 150 μm, respectively.

PKA. The data also imply that regulation of the cAMP signalling pathways in *S. cerevisiae* and *C. albicans* occur through similar mechanisms involving titration of free cAMP concentrations.

We envision several uses for the plasmid constructs. The main feature of the pHWP1GFP3 construct is its utility as a reporter of germ tube induction and true hyphae formation. Because *HWP1* expression is coordinately controlled with true hyphae formation (Staab *et al.*, 1996), *HWP1* mRNA has been used as a marker of germ tube formation (Braun & Johnson, 2000; Braun *et al.*, 2001; Davis *et al.*, 2002; Kadosh & Johnson, 2001; Lane *et al.*, 2001a, b; Liu, 2001; Murad *et al.*, 2001). The pHWP1GFP3 construct allows for the easy visualization of *HWP1*p activity in cells expressing true hyphae without having to prepare RNA for analysis. The customary method of *HWP1* mRNA analysis by Northern blotting only examines *HWP1* expression in a culture of cells without taking into account the percentage of germinating cells. The abundance of *HWP1* message in germinating cells (Staab *et al.*, 1996) makes its detection possible even if a small percentage of cells have germinated (data not shown). pHWP1GFP3-transformed strains permit easy determination of the percentage of germ tube formation in a mixed population of cells during the course of an experiment. In addition, GFP expression in true hyphae eliminates the guesswork of enumerating cells with very short germ tubes or cells that are forming pseudohyphae (negative for GFP).

A convenient feature of the plasmids is the ability to substitute yEGFP3 with heterologous genes for expression either concomitantly with germ tube induction or constitutively in all cell types. As an example we used the *ENO1*p plasmid to test the predicted germ-tube-defective phenotype resulting from overexpression of a member of the cAMP signalling pathway gene, *BCYI*, the regulatory subunit of PKA. The highly active *ENO1*p effectively overexpressed *BCY1* mRNA several fold relative to that driven by the native *BCY1*p (Fig. 5a). These results suggest that the pENO1GFP3 construct is amenable to other genetic studies such as epistatic analyses to determine the functional relationship of genes among signalling pathways. Alternatively, the *HWP1*p may be utilized for expressing genes in conjunction with germ tube formation. One caveat regarding the *HWP1* promoter is that it may not be the best choice for studying genes directly involved in dimorphism. Intermediate phenotypes may confuse the interpretation of results if the *HWP1* promoter is used to induce genes within filamentation signalling pathways that ultimately regulate *HWP1* expression.

The plasmids described here expand the molecular genetic tools for studying gene expression and functional relationships between gene products in *C. albicans*. Although the chromosomal integration of plasmids or DNA constructs for gene expression analyses in *C. albicans* have been described before (Backen *et al.*, 2000; Morschhauser *et al.*, 1998; Srikantha *et al.*, 1996; Uhl & Johnson, 2001), the visualization of GFP expression at the cellular level in tight association with morphology has not been reported. This makes the *HWP1*p construct attractive for studies examining true hyphae formation and filamentation, while the *ENO1*p permits constitutive overexpression of genes in all cell morphologies. Although it is difficult to assess the relative strengths of each promoter, both *HWP1* and *ENO1* express their cognate mRNAs at relatively high levels (Staab *et al.*, 1996, 1999). The brighter appearance of EGFP3 yeasts and germ tubes relative to HGFP3 germ tubes (data not shown) is most likely a result of the continuous accumulation of the stable GFP (Chalfie *et al.*, 1994; Li *et al.*, 1998) in EGFP3 cells. yEGFP3 mRNA is also detected in larger amounts in EGFP3 cells relative to HGFP3 germ tubes (data not shown), consistent with the constitutive expression of yEGFP3 by the *ENO1* promoter and apparent stability of the message. Nonetheless, both promoters express high amounts of GFP in *C. albicans* readily visible by epifluorescence. Both plasmids produce transformants with stable, low-copy integrations into the chromosome at a known genomic locus.

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