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APSES Proteins Regulate Morphogenesis and Metabolism in *Candida albicans*[D]

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Fungal APSES proteins regulate morphogenetic processes, including filamentation and differentiation. The human fungal pathogen *Candida albicans* contains two APSES proteins: the regulator Efg1p and its homologue Efh1p, described here. Overexpression of *EFG1* or *EFH1* led to similar phenotypes, including pseudohypha formation and *opaque-white* switching. An *efh1* deletion generated no phenotype under most conditions but caused hyperfilamentation in an *efg1* background under embedded or hypoxic conditions. This suggests cooperation of these APSES proteins in the suppression of an alternative morphogenetic signaling pathway. Genome-wide transcriptional profiling revealed that *EFG1* and *EFH1* regulate partially overlapping sets of genes associated with filament formation. Unexpectedly, Efg1p not only regulates genes involved in morphogenesis but also strongly influences the expression of metabolic genes, inducing glycolytic genes and repressing genes essential for oxidative metabolism. Using one- and two-hybrid assays, we further demonstrate that Efg1p is a repressor, whereas Efh1p is an activator of gene expression. Overall, the results suggest that Efh1p supports the regulatory functions of the primary regulator, Efg1p, and indicate a dual role for these APSES proteins in the regulation of fungal morphogenesis and metabolism.

INTRODUCTION

The APSES proteins represent a conserved class of transcriptional regulators that is unique to fungi, regulating cellular differentiation in the ascomycetes. In *Saccharomyces cerevisiae*, the Phd1 protein induces pseudohyphal growth, whereas the related Sok2 protein represses this growth form (Gimeno and Fink, 1994; Ward *et al*., 1996). The Asm1 protein promotes ascospore maturation in *Neurospora crassa* (Aramayo *et al*., 1996), and the StuA protein is required for conidiophore maturation in *Aspergillus nidulans* (Dutton *et al*., 1997). In the human fungal pathogen *Candida albicans*, the APSES protein Efg1p controls several morphogenetic processes. Efg1p regulates the yeast-to-hypha transition, it is required for the generation of chlamydospores, and it determines cell shape during *white-opaque* switching (Lo *et al*., 1997; Stoldt *et al*., 1997; Sonneborn *et al*., 1999a,b; Srikantha *et al*., 2000). Thus, all known APSES proteins regulate reversible interconversions between a spherical cell type (e.g., a budding yeast cell, conidiophore, ascospore, and chlamydospore) and an elongated filament (e.g., true hypha, pseudohypha, an *opaque*-form cell). In addition, as was shown for *C. albicans* Efg1p (Lo *et al*., 1997), APSES proteins may be involved in determining virulence of these organisms. However, the mechanisms by which APSES proteins stimulate cellular differentiation and regulate virulence are still unknown.

APSES proteins may act both as activators and repressors of gene expression because they stimulate reversible transitions between spherical and filamentous cells. The induction of true hyphae in *C. albicans*, by serum, for example, requires the presence of Efg1p. This led to the presumption that Efg1 acts as an activator of hypha-specific genes (Lo *et al*., 1997; Stoldt *et al*., 1997). Likewise, the generation of chlamydospores and of the *white* (yeast-like) cell form depends on Efg1p as a positive factor (Sonneborn *et al*., 1999a,b). However, *EFG1* expression is rapidly repressed by negative autoregulation after the onset of hyphal development, and it has been shown that Efg1p, in conjunction with the Sin3p-dependent histone deacetylase complex, mediates this repression (Tebarth *et al*., 2003). In addition, *EFG1* overexpression inhibits the formation of true hyphae, inducing pseudohyphae instead (Stoldt *et al*., 1997; Tebarth *et al*., 2003). An alternative pathway of hypha formation, which is operative in embedded or microaerophilic conditions, is repressed by Efg1p (Brown *et al*., 1999; Sonneborn *et al*., 1999a; Giusani *et al*., 2002). Overexpression of *PHD1* in *S. cerevisiae* has the same effect as the deletion of its homologue *SOK2* (Gimeno and Fink, 1994; Ward *et al*., 1996). Pseudohyphal growth is induced suggesting that Phd1p is an activator and Sok2p a repressor of pseudohypha formation and that the conserved APSES domain does not determine regulatory specificity. Genetic interactions with elements of a mitogen-activated protein kinase pathway also support an activator function of Phd1p (Lo *et al*., 1997), whereas Sok2p is known to repress meiosis via a defined regulatory sequence (Shen-

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har and Kassir, 2001). Interestingly, heterologous expression of Efg1p in *S. cerevisiae* suggests both repressor and activator functions for this regulator. *EFG1* is able to complement a *sok2* mutation (Shenhar and Kassir, 2001) and at high expression levels *EFG1* induces pseudohyphal growth (Stoldt *et al*., 1997).

The multiple functions of Efg1p suggest that it might interact with coregulatory proteins that modulate its functional specificities. The conserved domain of ~100 residues in APSES proteins contains a basic helix-loop-helix (bHLH) motif, which might dimerize with another bHLH protein as a prerequisite for their binding to E-boxes (Nasi *et al*., 2001). The bHLH domain of Efg1p has indeed been shown to bind E-box sequences in vitro (Leng *et al*., 2001), although in vivo gene regulation by an E-box motif has not been demonstrated for any APSES protein. Organisms often contain pairs of APSES proteins, for example, Phd1p and Sok2p in *S. cerevisiae*. Hence, we reasoned that a second APSES protein might exist in *C. albicans* and that this protein might modulate Efg1p function. Here, we report the identification of an Efg1p-homologue, Efh1p. We show that Efh1p does not have an obvious opposing role to Efg1p, as has been reported for Phd1p and Sok2p in *S. cerevisiae* (Gimeno and Fink, 1994; Ward *et al*., 1996). In contrast, Efh1p seems to modulate some of the functions of the main regulator Efg1p, which on a molecular level has the activity of a transcriptional repressor. We show that Efg1p and Efh1p not only regulate fungal morphogenesis but also favor a fermentative mode of metabolism of *C. albicans*. These results suggest that APSES proteins may coordinate morphogenetic and metabolic processes in fungi.

MATERIALS AND METHODS

Strains and Media

Strains are listed in Table 1. To isolate strains homozygous at the *MTL* locus, we isolated derivatives able to grow on sorbose as their sole carbon source (Magee and Magee, 2000). The identity of strains as *MTLa* was established by colony polymerase chain reaction (PCR) (primers MTLa-For, 5′-TTGAAGCGTGAGAGGCTAGGAG-3′; MTLa-Rev, 5′-ATCAATTCCCTTTCTCTTCGATTAGG-3′) or as MTLa (primers MTLα-For, 5′-TTCGAGTACATTCTGGTCGCG-3′; MTLα-Rev, 5′-TGTAAACATCCTCAATTGTACCCGA-3′). Phenotypic switching in homozygous *MTL* strains was monitored after pregrowth on modified Lee's medium (Lee *et al*., 1975), followed by plating on SC medium containing 5 μg/ml phloxine B and growth at 25°C for 7 d (Miller and Johnson, 2002). Strains were grown in YPD, SC, SCAA or on supplemented SD minimal medium (Sherman *et al*., 1986); YPS medium is identical to YPD but contains 2% sucrose as carbon source. S4D medium is SD medium containing 4% glucose. Serum plates contained 10% horse serum and 2% agar. Transformation was done by the spheroplast method (Sherman *et al*., 1986). To fully induce the *PCK1* promoter cells were grown in SCAA medium for several generations to a final OD_{600} = 0.8–1.0 (Stoldt *et al*., 1997). For growth in microaerophilic conditions, plates were placed in an anaerobic jar by using CampyGen bags (Oxoid, Basingstoke, Hampshire, England) to generate an atmosphere of ~6% oxygen. Cells were embedded in YPS-Agar essentially as described previously (Brown *et al*., 1999) except that a thin layer of YPS-agar (8 ml) was poured onto the cell-containing layer. This procedure prevented spreading of colonies on the agar surface and, by slowing filamentation, enhanced morphogenetic differences between *efg1* single and *efg1 efh1* double mutants.

EFH1 Deletion

The 5′region of *EFH1* was PCR amplified using primers EFH-FLP1 (5′-TGG GTA CCG GCC TGA TTA GAA TAT GAT TTC CG-3′; *Kpn*I, underlined) and EFH-FLP2 (5′-AAC TCG ACC GAG TGA CAA ACT AAT AGC AGAC-3′) and inserted into pUC18 (SureClone; Amersham Biosciences UK), resulting in plasmid pDB36. Similarly, the 3′ region was PCR amplified using primers EFH-FLP3 (5′-ATGCGGCCGCGGCCAATTTTCTTCAAATATTCTGG-3′; *Not*I, underlined) and EFH-FLP4 (5′-CAG AGCTCCTGGACCTTTCCTACCTGGATTCTC-3′; *Sac*I, underlined) and subcloned to generate pDB37. A 1035 base pairs *Not*I/*Sac*I fragment from pDB37 was cloned into *Not*I/*Sac*I-digested pSFU1 (Morschhäuser *et al*., 1999) flanking the "Ura-flipper," resulting in pDB40. A 1043-base pair *Kpn*I fragment from pDB36 was cloned into the *Kpn*I site of pDB40 to generate pDB43. The 4.89-kb *Sac*I fragment from pDB43 was transformed into strain CAI4 to disrupt the first *EFH1* allele. Removal of the *FLP-URA* cassette was carried out as described previously (Morschhäuser *et al*., 1999) and the procedure was repeated to disrupt the second allele.

The Ura-blaster method was used to make a double *efh1 efg1* mutant (Fonzi and Irwin, 1993). First, the 1-kb *Kpn*I fragment from pDB36 was cloned into p5921 to generate pSKM49. The 3′ region of *EFH1* was PCR amplified using primers SKM-BH1-EFH3′-1 (5′-ATTGGATCCAATTTTCTTCAAATATTCTG-G-3′) and SKM-*Bgl*II-EFH3′-2 (5′-ATTAGATCTACCTGGATTCTCTCAAC-3′) (*Bam*HI, *Bgl*II, underlined) and cloned into pUC18, resulting in pSKM47. A 1-kb *Bam*HI/*Bgl*II fragment from pSKM47 was cloned into pSKM49 to construct pSKM50. The *Sac*I-*Sph*I fragment from pSKM50 was used to disrupt *EFH1* alleles in strain HLC67 (Lo *et al*., 1999). The genotype of each mutant was confirmed by southern blotting (see Supplemental Data, Figure 1).

EFH1 Plasmids

EFH1 was PCR amplified from genomic DNA by using primers AN1-BHI-ATG (5′-TTGGATCC*ATGAATGGTATTATGACG*-3′) and AN3-Stp-BHI (5′-TTGGATCC*GTTTATCATAATGTTTTGTG*-3′ (*Bam*HI site, underlined; coding region, italics). The PCR fragment was cloned into pUC18 to generate pDB30. *EFH1* overexpression plasmids were constructed by insertion of the *EFH1* *Bam*HI-fragment downstream of the *PCK1* promoter into the *Bgl*II site of plasmid pBI-1 (Stoldt *et al*., 1997) or of plasmid pBT-44 to generate pDB35 (*URA3*) and pBT-145 (*ADE2*), respectively. To express an HA-tagged version of Efh1p the *Bam*HI fragment of pDB30 was subcloned first into the *Bam*HI site of YCpIF17 (Foreman and Davis, 1994) and the *HA-EFH1* fusion of this plasmid (pTD11) was subsequently amplified by primers HAEFH1(ATG)+*Hinc*II (5′-TTAGTTAACTTATATGAGTCGATACCCATAC-3′) and HAEFH(STOP)+HincII(5′-TTAGTCAACTTCATAATGTTTTGTGAAC-3′) (*Hinc*II, underlined). The *Hinc*II-digested PCR-fragment was cloned into the filled-in *Bgl*II site of pBI-1 to generate pTD13.

To join the *EFH1* promoter to the *RLUC* reporter, we amplified the 2-kb genomic region upstream of the *EFH1* open reading frame (ORF) by PCR from genomic DNA of strain SC5314, by using primers EFH1p-5 (5′-TTAGGATCCAGTTTACCCGAAATCTGTG-3′) and EFH1p-3 (5′-TTAGGATCCGTGAATATACTTATAACGAG-3′) (*Bam*HI, underlined). The *Bam*HI-digested PCR fragment was cloned into pUK21; into the *Spe*I site of the resulting plasmid pLR3 we inserted the 1-kb *Nhe*I-*Xba*I fragment *RLUC* fragment of pRL-Null (Promega, Madison, WI) to construct pLR4. The 3.1-kb *Spe*I fragment of pLR4 was then inserted into the *Xba*I site of the *URA3*-plasmid p1367/1 (Losberger and Ernst, 1989), resulting in pLR6. pLR6 was integrated into the genomic *EFH1* promoter by digestion with *Bst*I 107I and transformation of strain CAI4. For comparison an integrating *URA3* plasmid, pBT89a, containing a fusion of the major *EFG1* promoter to *RLUC* (Tebarth *et al*., 2003) was cut with *Hpa*I and integrated into the *EFG1* promoter of strain CAI4.

Transcriptional Profiling

DNA microarrays containing 6039 *C. albicans* ORFs of strain SC5314 (~98% of total) in duplicate were purchased from Eurogentec (Seraing, Belgium). Transcriptional profiling was performed on 50 ml of cells grown in YPD or SCAA medium at 30°C to an $OD_{600\ nm}$ = 0.5; alternatively, cells were pregrown in YPD medium at 30°C, starved in water for 1 h at 30°C, and then diluted and incubated in 10% horse serum at 37°C for 30 min (germ tubes were beginning to form at this time point). The isolation of total RNA, preparation of Cy3- and Cy5-cDNA and pairwise hybridization to DNA microarrays were performed according to Galar Fungail standard operating procedures (www.pasteur.fr/recherche/unites/Galar_Fungail/) with minor modifications. cDNAs were purified by the Qiaquick PCR purification kit (QIAGEN, Valenica, CA) and eluted from columns by twice rinsing with 50 μl of water (42°C). The eluate was concentrated using Microcon-YM30 columns (Millipore, Billerica, MA) to a volume of 10 μl. Ten microliters each of Cy3- and Cy5-labeled cDNA and herring sperm DNA (10 mg/ml) were mixed, denatured at 95°C for 2 min, and the quickly chilled on ice. Eighty microliters of hybridization mixture was added, and the solution was allowed to float onto DNA microarrays slides, which were covered with a rimmed cover glass (Erie Scientific, Portsmouth, NH). Slides were placed in a hybridization chamber (Corning, Palo Lato, CA) and hybridized for 24 h at 42°C by immersion in a water bath. After washing and drying, microarrays were scanned by a FLA-8000 scanner (Fuji, Tokyo, Japan) at a resolution of 10 μm. Cy3- and Cy5-fluorescence signals were measured at 532 and 635 nm, respectively. For quantitation of spot and background fluorescence the program AIDA Array Metrix (Raytest, Pittsburgh, PA) was used.

Data from at least three independent experiments using independent cultures and including one dye-swap experiment were evaluated with the GeneSpring (Silicon Genetics, Redwood City, CA) software. The Cy3-/Cy5-ratios were first normalized based on the overall fluorescence intensity and then exported into Excel spreadsheets. Because each ORF was spotted twice on each array and at least three independent experiments were carried out, at least six signal intensity ratios reflecting the activity of a test sample to a reference sample were compared. The data were statistically analyzed using the SAM program (www-stat.stanford.edu/~tibs/SAM), which calculated q-values reflecting variation among the six ratios (a low q-value indicates a low level of variation). The predicted minimal

Table 1. Strains and plasmids

Strain or plasmid	Genotype or description	Reference/source
C. albicans strains		
SC5314	prototrophic	Fonzi and Irwin (1993)
CAF2-1		Fonzi and Irwin (1993)
CAF2-1/01α	as CAF2-1, but *MTLα/MTLα*	This work
CAF2-1/01α-op	as CAF2-1/01α, but *opaque* growth form	This work
CAI4	Δ*ura3*::*imm*434/Δ*ura3*::*imm*434	Fonzi and Irwin (1993)
CAI8	*ade2*::*hisG/ade2*::*hisG* Δ*ura3*::*imm*434/Δ*ura3*::*imm*434	Fonzi and Irwin (1993)
C4/c, C4/d	as CAI4, but *EFH1/efh1*Δ::*FRT-SAP2p-FLP-URA3-FRT*	This work
C4/c4, C4/d6	as CAI4, but *EFH1/efh1*Δ::*FRT*	This work
C4/d6-3	as CAI4, but *efh1*Δ::*FRT/efh1*Δ::*FRT-SAP2p-FLP-URA3-FRT*	This work
efh/02α	as C4/d6-3, but *MTLα/MTLα*	This work
efh/02α-op	as efh/02α, but *opaque* growth form	This work
C4/d63-1, C4/d63-3	as CAI4, but *efh1*Δ::*FRT/efh1*Δ::*FRT*	This work
JKC19	as CAI4, but *cph1*Δ::*hisG/cph1*Δ::*hisG-URA3-hisG*	Liu *et al.* (1994)
HLC52	as CAI4, but *efg1*Δ::*hisG/efg1*Δ::*hisG-URA3-hisG*	Lo *et al.* (1997)
Bca09-4	as CAI4, but *efg1*Δ::*hisG/efg1*Δ::*hisG-URA3-hisG*	Braun and Johnson (2000)
HLC67	as CAI4, but *efg1*Δ::*hisG/efg1*Δ::*hisG*	Lo *et al.* (1997)
HLC67[pDB35]a	HLC67 carrying pDB35, made *MTLa/MTLa*	This work
HLC74	as CAI4, but *efg1*Δ::*hisG/efg1*Δ::*hisG (EFG1)*	Lo *et al.* (1997)
HLC54	as HLC52, but *cph1*Δ::*hisG/cph1*Δ::*hisG*	Lo *et al.* (1997)
H/1.22, H/1.26	as HLC67, but *efh1*Δ::*hisG/efh1*Δ::*hisG-URA3-hisG*	This work
H/1.22-1, H/1.26-1	as HLC67, but *efh1*Δ::*hisG/efh1*Δ::*hisG*	This work
CRC101-103	as CAI8, but containing *ADH1-lacZ*::*ADE2*	Russell and Brown, unpublished data
CRC104-106	as CAI8, but containing *lexA-ADH1-lacZ*::*ADE2*	Russell and Brown, unpublished data
Red3/6	*ade2/ade2*, phenotypic switching strain	Srikantha *et al.* (2000)
Transformation vectors		
pRC18	*URA3*-marked *CARS2*-vector	Stoldt *et al.* (1997)
pBI-1	*PCK1*-promoter in *URA3*-marked *CARS2*-vector	Stoldt *et al.* (1997)
pAPE(2)/ADE	*PCK1*-promoter-*EFG1* fusion in *CARS2 ADE2*-vector	Sonneborn *et al.* (1999b)
pRC2312P-H	*PCK1*-promoter-*EFG1* fusion in pBI-1	Stoldt *et al.* (1997)
pDB35	*PCK1*-promoter-*EFH1* fusion in pBI-1	This work
pBT-4	*CARS2 ADE2*-vector	Sonneborn *et al.* (1999b)
pBT-44	*PCK1*-promoter in *CARS2 ADE2*-vector	This work
pBT-145	*PCK1*-promoter-*EFH1* fusion in pBT-4	This work
pTD13	*PCK1*-promoter-*HA-EFH1* fusion in pBI-1	This work
pBT-34	Major *EFG1*-promoter-*LAC4* fusion in *CARS2 URA3*-vector	Tebarth *et al.* (2003)
p89a	Major *EFG1*-promoter-*RLUC* fusion in *URA3*-vector	This work
pLR6	*EFH1* promoter-*RLUC* fusion in *URA3* vector	This work
CIp-LexA	*URA3*-marked integrating vector containing *ACT1p-lexA*	Russell and Brown, unpublished data
CIp-LexA-Efg1	as CIp-LexA, but containing *ACT1p-lexA-EFG1*	This work
CIp-LexA-Efh1	as CIp-LexA, but containing *ACT1p-lexA-EFH1*	This work
pGBD-C1	*TRP1* vector with *GAL4-BD*	James *et al.* (1996)
pGAD-C1	*LEU2* vector with *GAL4-AD*	James *et al.* (1996)
pDB17	pGBD-C1 containing *EFG1*	This work
pDB16	pGAD-C1 containing *EFG1*	This work
pDB34	pGBD-C1 containing *EFH1*	This work
pDB33	pGAD-C1 containing *EFH1*	This work
pSKM69	pGBD-C1 containing *efh1*ΔN1	This work
pSKM68	pGAD-C1 containing *efh1*ΔN1	This work
pCS5	pGBD-C1 containing *efh1*ΔN2	This work
pCS6	pGAD-C1 containing *efh1*ΔN2	This work
pCS7	pGBD-C1 containing *efh1*ΔN3	This work
pCS8	pGAD-C1 containing *efh1*ΔN3	This work

false discovery rate (FDR) value in SAM evaluations in most cases had a value close to 5% (see Supplemental Data, Tables 1–8). For comparisons of data sets, a delta value was chosen corresponding to an FDR value of 5%; in one case (strain HLC52 grown in YPD medium), >1000 regulated genes were predicted by this parameter, and a delta value corresponding to a FDR of 1% was chosen. For Venn diagrams and for gene listings, we applied more stringent criteria, defining genes as significantly regulated if they were regulated by at least a factor of 1.5-fold. Cluster analyses and Venn diagrams were carried out with the GeneSpring program, by using standard value settings. Gene designations were according to the annotation of the Candida DB Web server (http://genolist.pasteur.fr/CandidaDB/).

One- and Two-Hybrid Analyses

Details of the *C. albicans* one-hybrid system are described elsewhere (Russell and Brown, unpublished data). Briefly, expression vectors encoding *Staphylococcus* LexA protein fusions to Efg1p or Efh1p were constructed by PCR amplification of the *EFG1* or *EFH1* ORFs and inserting them into CIp-LexA, resulting in plasmids CIp-LexA-Efg1 and CIp-LexA-Efh1, respectively. The 5′ PCR primers used introduced a $(Gly)_3$-Pro-$(Gly)_2$ linker between the amino-terminal LexA domain and the carboxy terminal domains of Efg1p or Efh1p. These plasmids were introduced into *C. albicans* reporter strains that carried pCR-lacZ (no *lexA* operator upstream of basic *ADH1* promoter) or pCR-

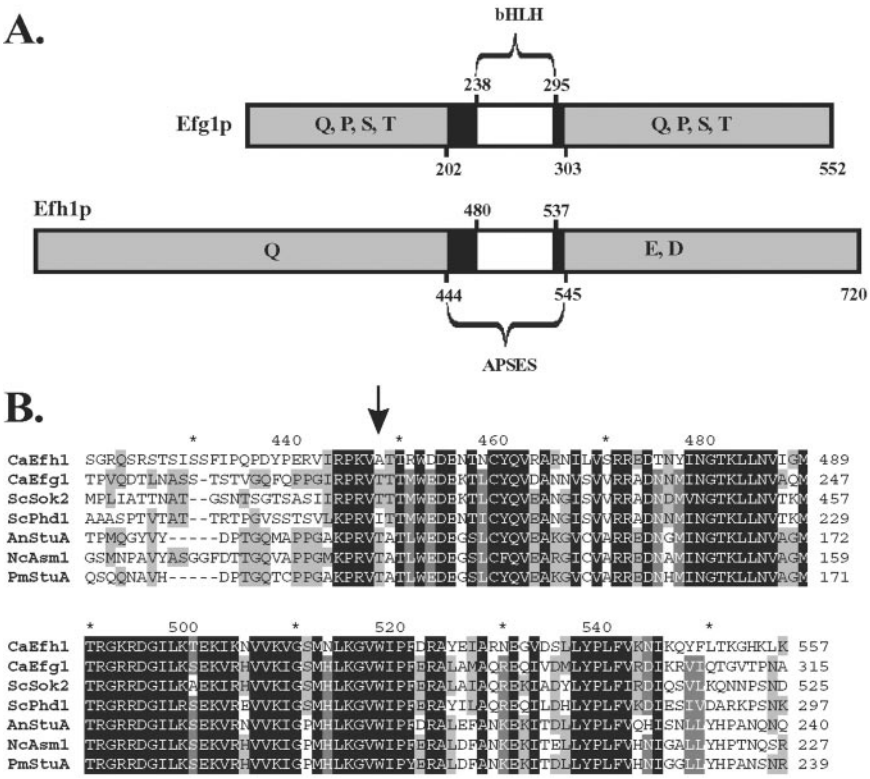

Figure 1. APSES proteins in *C. albicans.* (A) Schematic structures of Efg1 and Efh1 proteins. The location of bHLH and APSES domains and of regions enriched in the indicated amino acids is shown. (B) Comparison of APSES proteins. The arrow indicates the position of a potential phosphorylation site in Efg1p and Sok2p, which is absent in Efh1p and Phd1p. Proteins from the following species are compared: *C. albicans* (Efh1p/Efg1p), *S. cerevisiae* (Phd1p/Sok2p), *A. nidulans* (StuA), *N. crassa* (Asm1), and *P. marneffei* (StuA). Black boxes, identical residues; gray boxes, similar residues in a subset of APSES proteins.

OplacZ (containing *lexA* operator) integrated into the *ade2*::*hisG* locus (Russell and Brown, unpublished data).

For two-hybrid experiments, plasmids encoding protein fusions to the Gal4p-DNA-binding domain (BD) or the Gal4p-transcriptional activation domain (AD) were constructed by inserting appropriate DNA fragments into plasmids pGBD-C1 and pGAD-C1, respectively (James *et al*., 1996). The 1.5-kb *Eco*RI/*Bam*HI fragment of pUC19-EFG1 was used to construct *EFG1* expression vectors pDB17 (BD) and pDB16 (AD). Similarly, the 2.1-kb *Bam*HI fragment of pDB30 was used to construct *EFH1* vectors pDB34 (BD) and pDB33 (AD). Subfragments of the pDB30 insertion fragment also were cloned into pGBD-C1 or pGAD-C1 to test the effect of C-terminal shortening of Efh1p: (a) deletion *efh1*ΔN1: the *Bam*HI-*Bgl*II fragment generated pSKM69 (BD) and pSKM68 (AD); (b) deletion *efh1*ΔN2: the *Bam*HI-*Pst*I fragment generated pCS5 (BD) and pCS6 (AD); and (c) deletion *efh1*ΔN3: the *Bam*HI-*Sca*I fragment generated pCS7 (BD) and pCS8 (AD). Plasmids were transformed pairwise into strain PJ69–4A (*MATa trp1-901 leu2,3-112 ura3-52 his3-200 gal4*Δ *gal80*Δ *LYS2*::*GAL1-HIS3 GAL2-ADE2 met2*::*GAL7-lacZ*) and assayed for prototrophy for histidine and adenine, as well as for β-galactosidase (LacZ) activity (James *et al*., 1996).

Reporter Gene Assays

RLUC activity was determined using the Dual-Luciferase Reporter Assay (Promega). To 50–100 μl of LARII reagent 10 μl of crude extract was added and after 10 s at room temperature, 50–100 μl of Stop&Glo reagent was used to stop the reaction. Immediately afterward, light emission was assayed in a luminometer (Fluorskan Ascent FL; Labsystem, Helsinki, Finland). β-Galactosidase activity in cells, expressed in Miller units, was determined by standard procedures on plates containing X-Gal or in liquid by using *O*-nitrophenyl β-D-galactopyranoside (ONPG) as indicators (Miller, 1972).

RESULTS

EFH1 Is Related to EFG1

Analysis of *C. albicans* genomic sequences (http://www.sequence.stanford.edu/group/candida) by using the BLAST algorithm revealed a single *EFG1* homologue (orf6.4659), which we named *EFH1* (*EFG1* homologue). The *EFH1* ORF is 45% identical to the *EFG1* ORF, and it encodes a hypothetical protein of 720 residues (81,373 Da), which is 23% identical to the 552 aa-Efg1 protein. Efh1p displays high similarity to Efg1p and other APSES proteins within a domain encompassing residues 443–546 (65% amino acid sequence identity) (Figure 1). This APSES domain contains a bHLH subdomain from residues 480–537. Efh1p lacks the putative protein kinase A phosphorylation target in the APSES domain corresponding to residue 206 in Efg1 (Bockmühl and Ernst, 2001). Outside the APSES domain, Efh1p and Efg1p share little sequence similarity except for the occurrence of glutamine-rich stretches. In Efh1p, these occur at residues 85–102 and 200–223. A unique feature of Efh1p is a stretch of acidic residues between residues 615 and 643.

Northern analyses revealed two low abundance *EFH1* transcripts of ~2.5 and 2.7 kb (our unpublished data), indicating that *EFH1* is expressed at low levels. To compare the transcriptional activities of *EFH1* and *EFG1* loci, we integrated the *RLUC* reporter in place of the respective ORFs, by chromosomal integration of plasmids pLR6 and p89a to generate *EFH1p-RLUC* and *EFG1p-RLUC* fusions, respectively. Luciferase activities were ~10-fold lower for the *EFH1p-RLUC* fusion compared with the *EFG1p-RLUC* fusion during growth in minimal medium (0.003 ± 0.001 vs. 0.025 ± 0.002 relative light units/μg protein, respectively). This suggests that the *EFH1* promoter has an ~10-fold lower activity compared with the *EFG1* promoter under these conditions.

Phenotypes of efh1 Mutants

A homozygous *efh1/efh1* mutant was constructed to examine the function of *EFH1* (strain C4/d6-3). In addition, heterozygous *EFH1/efh1* strains were obtained in this process (strains C4/c, d). The growth characteristics of these mutants were identical to the isogenic wild-type strain under numerous conditions, and no obvious antifungal resistance or stress phenotypes were detected. The cellular morphologies of *efh1*

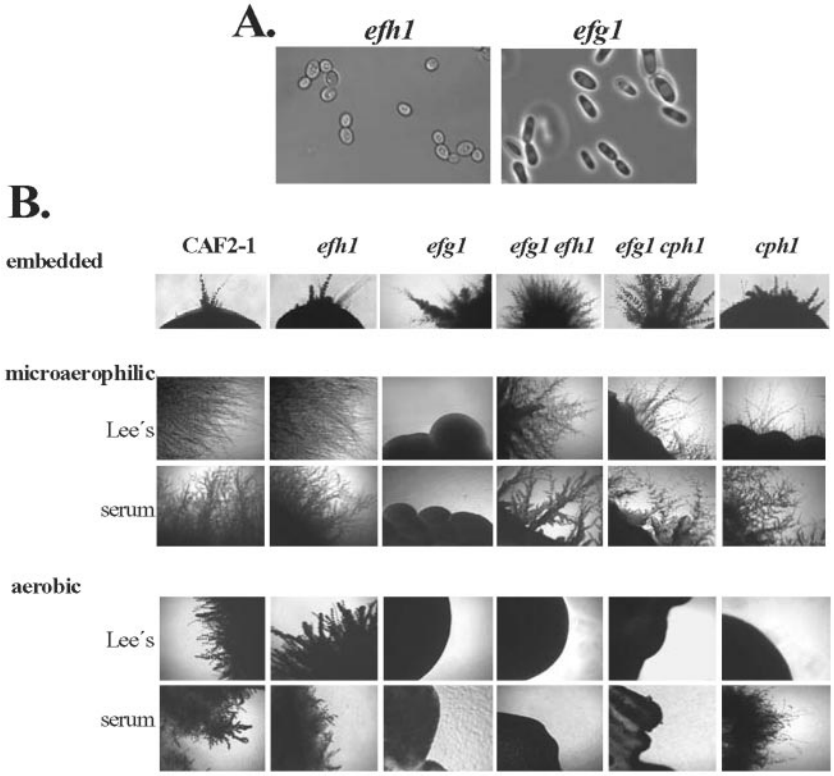

Figure 2. Morphological phenotypes of *efh1* and *efg1* mutants. (A) Cellular morphology of an *efh1* mutant (strain C4/d63-1) and an *efg1* mutant (strain HLC52) visualized by phase contrast microscopy. (B) Comparison of colony phenotypes. Strains CAF2-1 (wt), C4/d63-1 (Δ*efh1*), HLC52 (Δ*efg1*), H/1.22 (Δ*efh1* Δ*efg1*), HLC54 (Δ*efg1* Δ*cph1*), and JKC19 (Δ*cph1*) were grown for 5 d at 24°C embedded in agar or at 30°C in a jar generating microaerophilic conditions or aerobically.

mutants were similar to the wild-type strain, in contrast to the rod-like shape of *efg1* mutants (Figure 2A). Hyphal development was normal on solid and in liquid media (Lee's, Spider, low-nitrogen and serum-containing media) and under embedded conditions (see below). In addition, chlamydospore formation was normal in cells lacking Efh1p. To test whether the inactivation of Efh1 effected phenotypic switching, we measured *white-opaque* switching in the homozygous *MTLα/MTLα efh1/efh1* strain efh/02α. Isogenic wild-type and *efh1* cells switched from *white* to *opaque* at a frequency of ~5% as expected (Miller and Johnson, 2002). Likewise, *opaque* cells that arose in this experiment frequently switched back to the *white* form (our unpublished data). Thus, switching occurred normally in *efh1* cells, in contrast to *efg1* cells that seemed locked in an *opaque*-like state (Sonneborn *et al*., 1999b). Collectively, our data indicate that an *efh1* mutation does not influence the various morphogenetic processes that are strongly affected by an *efg1* mutation (morphogenesis and phenotypic switching).

Sok2p and Phd1p have opposing regulatory functions in *S. cerevisiae*. Hence, we examined the possibility of genetic interactions between *EFH1* and *EFG1* by comparing the phenotypes of *efh1* and *efg1* single mutants with *efh1 efg1* double mutants (strains C4/d63-1, HLC67, and H/1.22). During growth in standard hypha-inducing conditions the *efh1 efg1* double mutant showed similar defects in hypha and chlamydospore formation to an *efg1* single mutant, generating the rod-like cells typical of an *efg1* mutant (our unpublished data).

We further tested morphogenesis under hypoxic or embedded conditions, in which Efg1p seems to repress a poorly defined alternative signaling pathway regulating hypha formation (Brown *et al*., 1999; Sonneborn *et al*., 1999a). When *C. albicans* cells were grown embedded in agar at 24°C, *efg1* cells displayed increased hypha formation compared with isogenic wild-type or *efh1* cells (Figure 2B). In contrast, *efg1* cells did not form hypha in a jar generating microaerophilic conditions (~6% oxygen), whereas *efh1* cells formed hyphae like the wild-type parent under these conditions (Figure 2B).

We observed a synthetic phenotype between *efh1* and *efg1* mutations with regard to filament formation during growth under some embedding conditions (see MATERIALS AND METHODS). Compared with the *efh1* and *efg1* single mutants, the *efh1 efg1* double mutants formed colonies with lateral hyphae faster and filamentation was more extensive. Furthermore, under microaerophilic conditions the *efg1 efh1* double mutant filamented vigorously, like wild-type and *efh1* cells, whereas the *efg1* single mutant did not filament; also, in these conditions, an *efg1* mutation did not block filamentation occurring in an *cph1* genetic background. (Figure 2B). We interpret this to mean that the simultaneous inactivation of Efg1p and Efh1p (or Cph1p) derepresses an alternative pathway of filamentation in an oxygen-dependent manner. Under aerobic conditions Efg1p is required to form hyphae. However, this requirement for Efg1p is released by the additional inactivation of Efh1p, but only under low-oxygen or embedding conditions. In more hypoxic conditions (i.e., in a stream of nitrogen), however, the alternative pathway was almost fully induced in an *efg1* mutant, and its excessive filamentation was not greatly increased by an *efh1* mutation (our unpublished data). Thus, it seems that an *efh1* phenotype is observed especially in intermediate conditions, in which neither the standard nor the alternative pathways of filamentation are fully induced.

EFH1 Overexpression Phenotypes

The deletion of the *EFH1* locus alone had no obvious effect upon morphogenesis in *C. albicans*. Hence, we investigated whether *EFH1* overexpression would have an effect on this

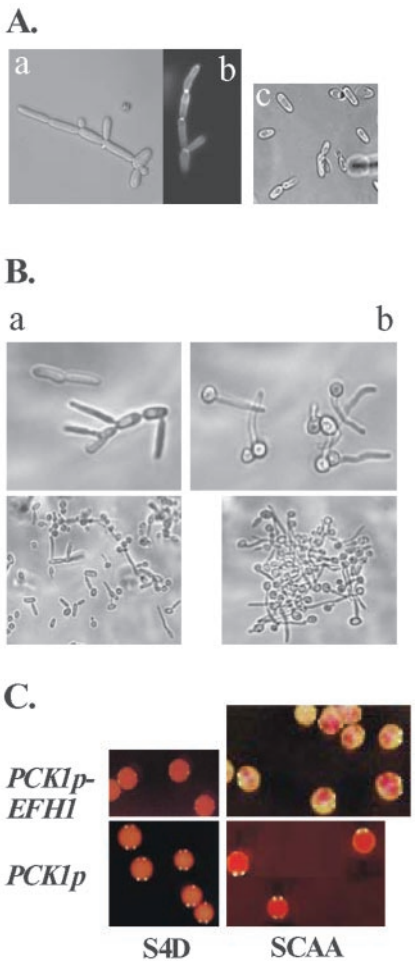

Figure 3. Phenotypes of *EFH1* overexpression. (A) Generation of pseudohyphae. A pDB35-transformant of strain CAI4 (a and b) or of *efg1* mutant HLC67 (c) was grown in SCAA medium before microscopy. Intercellular junctions are visualized by calcofluor white fluorescence (b). (B) Inhibition of true hypha formation. A pDB35-transformant of strain CAI4 was pregrown in SCAA medium (a) or SD medium (b) and shifted to the same medium containing 10% serum and grown for 60 min at 37°C. At low microscopic magnification (bottom photographs), the aggregate-formation by hyphae of SD-grown cells is visible. (C) Promotion of *opaque* to *white* phenotypic switching. The *opaque* form of strain Red3/6 was transformed with pBT-145 (*PCK1p-EFH1*) or with control vector pBT-44 (*PCK1p*) and transformants were grown at 25°C on *PCK1p*-repressing S4D or -inducing SCAA medium containing phloxine B. Note the speckled appearance of pBT-145 transformants on SCAA plates consisting mainly of the *white* form but containing red sectors indicative of the *opaque* form.

process. Strains expressing *EFH1* under the regulatable *PCK1* promotor grew as regular or slightly elongated yeast cells under repressing conditions, but they formed pseudohyphae after induction of the *PCK1* promoter. These cells budded apically and had calcofluor white-stainable constrictions at cellular junctions (Figure 3A). Interestingly, *EFH1* overexpression did not lead to pseudohyphal formation in an *efg1* genetic background (HLC67) (Figure 3A, c). However, *EFG1* overexpression produced pseudohyphae in both an *efg1* and an *efh1* background (our unpublished data). Thus, although overexpression of either *EFG1* or *EFH1* induces pseudohyphal development, Efg1p would seem to be the main regulator of this process, because the *EFH1*-overexpression phenotype requires the presence of Efg1p.

We showed previously that *EFG1* overexpression blocks the formation of true hyphae (Tebarth *et al*., 2003). Interestingly, *EFH1*-overexpressing cells generated the same phenotype: pseudohyphal cells incapable of forming true hyphae in response to serum (Figure 3B). In contrast, cells expressing *EFH1* at low levels did respond to serum, forming strongly aggregating germ tubes and true hyphae. Hypha production was still blocked in *efg1* cells overexpressing *EFH1*, indicating that the requirement for Efg1p cannot be bypassed by *EFH1* overexpression.

EFG1 overexpression causes rapid phenotypic switching from *opaque* to *white* cells (Sonneborn *et al*., 1999b). To test whether *EFH1* overexpression affects this process, we examined the behavior of *opaque* Red3/6 cells carrying a *PCK1p-EFH1* fusion (pBT-145) on media containing phloxine B (which stains *opaque* colonies pink, whereas *white*-form colonies are white). All of the cells formed pink colonies on *PCK1p*-repressing S4D-medium indicating that the *opaque* form was stable at low *EFH1* expression levels (Figure 3C). Control transformants grown on SCAA medium remained purely *opaque*. In contrast, *EFH1* overexpression on SCAA medium generated mostly white colonies, although some pink spots indicative of *opaque* cells did remain (Figure 3C). Thus, *EFH1* overexpression was able to induce the *opaque* to *white* conversion, whereas it did not affect the reverse switching process (*white-opaque*) (our unpublished data). To test whether forced *opaque* to *white* conversion was dependent on Efg1p, we overexpressed *EFH1* in the *MTLa efg1*-homozygous strain (HLC67[pDB35]a), which on phloxine plates formed *opaque*-like pink colonies (Sonneborn *et al*., 1999b). In this case, overexpression of *EFH1* did not give rise to *white* form colonies. We conclude that *opaque* to *white* switching, which is triggered by *EFH1* overexpression, is dependent on Efg1p.

Transcriptomes of Strains Lacking APSES Proteins

Transcript profiling was used to examine further the roles of the APSES proteins in *C. albicans*. DNA microarrays carrying an almost complete set of *C. albicans* ORFs were used for these experiments, in which the transcriptomes of *efg1* and *efh1* mutants were compared with wild-type cells under identical growth conditions. Genes that displayed reproducible and statistically significant changes in expression relative to wild-type cells were identified after exposure to hypha-inducing or -noninducing growth conditions (Figure 4A). Lists of all genes that displayed significant regulation by Efg1p and Efh1p are provided as supplemental data (Supplemental Data, Tables 1–6).

To explore the functions of the APSES proteins under conditions that promote growth in the yeast form, we compared the transcriptomes of wild-type and mutant strains in YPD medium at 30°C. The deletion of *EFG1* altered the expression of 283 genes by >1.5-fold, with 100 genes being up-regulated and 183 being down-regulated. Only nine genes were affected by the deletion of *EFH1* (8 genes were up- and 1 was down-regulated) (Figure 4A). Only two genes of unknown function (*IPF4696* and *IPF4328*) were commonly affected in both single mutants. The simultaneous deletion of *EFG1* and *EFH1* showed a transcriptional pattern that overlapped with that of *efg1* single mutants (49 genes). However, a new subset of 63 genes was affected in the *efg1 efh1* double mutant that was not affected in the *efg1* single mutant. This is consistent with the existence of synthetic interactions between *EFG1* and *EFH1*. These results indicate

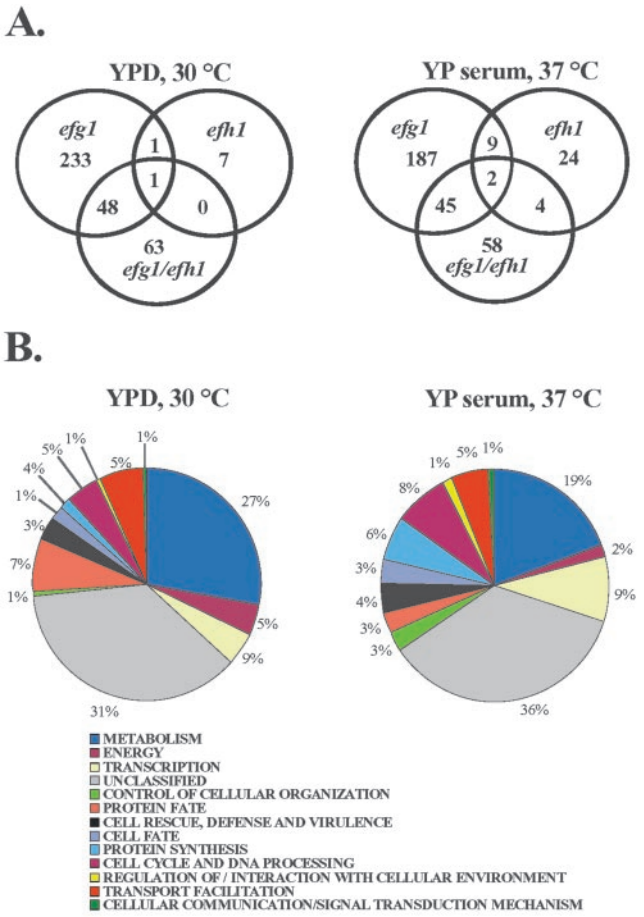

Figure 4. Transcript profiles of *C. albicans* mutants lacking the APSES proteins Efg1p or Efh1p. (A) Venn diagrams of genes regulated by Efg1p and Efh1p. The wild-type strain CAF2–1 was used as reference for the *efg1* mutant (HLC52), the *efh1* mutant (C4/d6-3) and the *efg1 efh1* double mutant (H/1.22), which were all grown in identical conditions: YPD/30°C (non-inducing) or for 30 min in hypha-inducing YP serum, 37°C. (B) Functional categories of genes regulated by Efg1p in the indicated conditions.

that under standard growth conditions Efg1p (but not Efh1p) has an important role in gene regulation.

Categorization of the regulated genes according to their functional categories of the MIPS database (http://mips.gsf.de/genre/proj/yeast/searchCatalogFirstAction.do?db=CYGD) revealed that deletion of *EFG1* alone or in combination with an *EFH1* deletion had a striking effect on carbon metabolism (Figures 4B and 5; Supplemental Data, Table 2). Transcript levels for almost all glycolytic genes (e.g., *FBA1*, *PFK1*, and *ENO1*) and for genes involved in the metabolism of reserve sugars trehalose and glycogen (e.g., *TPS2*, *TPS3*, and *GSY1*) were reduced in an *efg1* mutant by a factor of 2 or 3. In addition, transcript levels for several citric acid cycle enzymes (e.g., *FUM12* and *KGD1*), enzymes of the mitochondrial ATPase complex, and glycine and formate catabolic enzymes were increased (Figure 6). In contrast, the inactivation of Efh1p affected few glycolytic genes only slightly (Figure 5 and Supplemental Data, Table 2).

To confirm the transcriptional profiling data, we performed Northern analysis on a selection of Efg1p-regulated genes (Figure 6). The results confirmed the reduced levels of two glycolytic transcripts in the *efg1* mutant (*FBA1* and *PFK1*) and the increased levels of the *HHF22* and *FUM12* transcripts in the *efg1* mutant (Figure 6E).

To verify the biological consequences of a presumed reduced glycolytic flux in *efg1* strains, we compared its growth to a wild-type strain in the presence of antimycin A, which blocks ATP production by the respiratory chain. We indeed observed that two independently constructed *efg1* mutants (strains Bca09-4 and HLC52) grew similar to the wild-type in medium without antimycin A, but in the presence of antimycin A were retarded in growth (Figure 7). Insertion of a single wild-type copy of *EFG1* into the mutant background of strain HLC52 (strain HLC74) partially increased antimycin A-resistance as expected from a reduced *EFG1* gene dosage (Fu *et al*., 2002). To confirm the enhanced antimycin A-sensitivity of the *efg1* mutants, we also performed a mixing experiment, in which the wild-type CAF2–1 and the *efg1* mutant HLC52 were grown in the same YPD culture. At the starting $OD_{600\ nm}$ of 0.05 *efg1* mutant cells amounted to 60% of cells in the mixed culture and slightly decreased to 40% after 12 h of growth (final $OD_{600\ nm}$ = 12). In contrast, growth in the presence of 100 μM antimycin A for 12 h led to an strong decrease of *efg1* mutant cells to 10% (final $OD_{600\ nm}$ = 5). These results are consistent with reduced glycolytic ATP-production in *efg1* mutants, suggesting a dual function of Efg1p in morphogenesis and metabolism.

Other genes regulated by Efg1p under noninducing conditions include genes encoding histones, stress response-proteins membrane transporters, and putative cell wall proteins (Figure 5). Another interesting group of genes that seems to be repressed by Efg1p encode for putative transcriptional activators (*CTA21*, *CTA24*, and *CTA26*), raising the possibility that Efg1 is a global regulator of gene expression. The highest Efg1p-dependent regulation (30-fold upregulation) was observed for a putative multidrug resistance protein (*IPF14540*) (Supplemental Data, Table 1).

Transcriptomes during Morphogenesis

In a previous study, the transcriptomes of wild-type and *efg1* strains were compared after growth in hypha-inducing conditions for several generations (Nantel *et al*., 2002). In this study, we focused upon the identification of genes whose expression depends on the APSES proteins during an early stage of hyphal development. We generated transcriptional profiles for *efg1* and *efh1* cells exposed to serum for only 30 min at 37°C, by using a wild-type strain (CAF2–1) as reference. At this time point ~50% of wild-type cells showed short protrusions, which developed into germ tubes and ultimately formed hyphae during prolonged incubation. Under these hypha-inducing conditions, the deletion of *EFG1* caused major changes in the transcriptome. The expression of 243 genes was affected by a factor of >1.5 in *efg1* cells compared with wild-type cells. In contrast, the deletion of *EFH1* had a relatively small effect, affecting the expression of 39 genes (Figure 4A). However, 58 new genes were affected by the *efg1 efh1* double mutant, supporting the idea that *EFG1* and *EFH1* interact synthetically to regulate the expression of some *C. albicans* genes.

Again, glycolytic genes were expressed at lower levels in cells lacking Efg1p (Figures 4B and 5). However, in contrast to the analysis under yeast growth conditions, genes involved in oxidative metabolism were not upregulated in *efg1* cells during hyphal development (e.g., *CIT1*, *MDH1*, *KGD1*, *FDH3.3F*, *FDH4.3F*, and *FDH12*) (Figure 5C). Thus, Efg1p seems to repress these genes under noninducing conditions, but not during hyphal development. This pattern might be due to the lack of glucose in the inducing medium used.

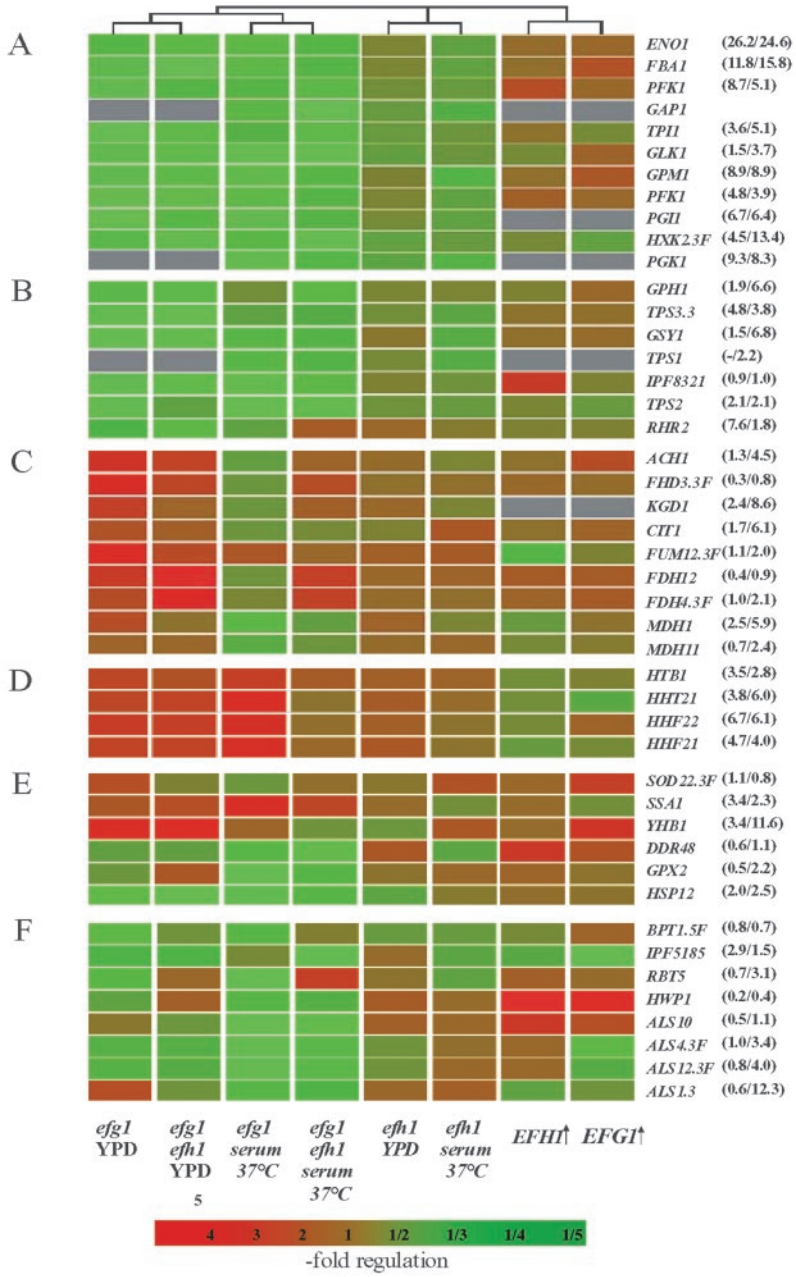

Figure 5. Transcriptional regulation of individual genes by Efg1p or Efh1p. Representative genes responsible for specific cellular functions are grouped and their regulation in *efg1* and *efh1* single and double mutants and strains overexpressing *EFG1* or *EFH1* relative to the CAF2-1 wild-type strain is listed. Genes involved in glycolysis (A), reserve sugar metabolism (B), citric acid cycle (C), histone biosynthesis (D), stress response (E), and cell wall biosynthesis (F) are compared. Numbers next to gene designations indicate the relative transcript levels of such genes (transcript level of individual gene relative to the average level of all genes) in the wild-type strain CAF2-1, grown in YPD (30°C) and YP serum (37°C), respectively.

Therefore, Efg1p-dependent repression of genes involved in oxidative metabolism might require the presence of glucose.

Transcript Profiling of EFG1- or EFH1-Overexpression

To identify genes that are regulated by high levels of APSES proteins, we performed transcript profiling of *C. albicans* cells overexpressing *EFG1* (pRC2312P-H) or *EFH1* (pDB35) from the *PCK1* promoter. Cells containing the empty expression vector (pBI-1) were used as reference. The transcript levels of 85 and 81 genes were affected by elevated *EFG1* or *EFH1* expression, respectively (Figure 8; Supplemental Data, Tables 7 and 8).

Among the 85 genes regulated by *EFG1* overexpression, 53 genes were down-regulated (1.5- to 3.5-fold) and 32 genes were up-regulated (1.5- to 19-fold). The up-regulated genes encode cell wall proteins such as Hwp1p, Als10p, Rbt5p, Ece1p, and Phr1p, which are known to be associated with filamentous growth in *C. albicans* (Birse *et al*., 1993; Bailey *et al*., 1996; Fonzi, 1999; Sharkey *et al*., 1999; Braun and Johnson, 2000; Braun *et al*., 2000; Lane *et al*., 2001) (Figure 5F). The *DDR48* gene, encoding a stress protein, and *IPF1222* (*SOD5*), encoding a hypha-specific superoxide dismutase (Nantel *et al*., 2002), also were up-regulated. Most of these genes make up the overlap of 14 genes reduced by the absence of Efg1p in inducing conditions, and induced by *EFG1* overexpression (Figure 8A). Whereas *EFG1* down-regulated a majority of genes, *EFH1* overexpression up-regulated most genes (53 up-regulated and 28 down-regulated genes). In agreement with the related phenotypes generated by *EFG1* and *EFH1* overexpression, the corresponding transcriptional profiles were similar (Figure 8B). Especially filament-specific genes such as *HWP1* and *ECE1* but also metabolic genes such as

Table 2. Regulation of genes encoding metabolic enzymes

Gene	Accession no.	Description (CandidaDB)	Fold regulation		
			efg1	*efg1-efh1*	*efh1*
Glycolysis					
ENO1	CA3874	Enolase I	0.66	0.60	0.96
FBA1	CA5180	Fructose-bisphosphate aldolase	0.37	0.46	0.99
GLK1	CA0263	Aldohexose specific glucokinase	0.40	0.54	0.80
GPM1	CA4671	Phosphoglycerate mutase	0.40	0.56	0.99
PFK1	CA1834	Phosphofructokinase, alpha subunit	0.46	0.53	0.97
PFK2	CA3112	Phosphofructokinase, beta subunit	0.41	0.65	0.93
PGI1	CA3559	Glucose-6-phosphate isomerase	0.50	0.64	0.96
TPI1	CA5950	Triose phosphate isomerase	0.44	0.58	0.82
HXK2.3F	CA0127	Hexokinase II	0.36	0.49	0.76
Reserve carbohydrates					
TPS2	CA5066	Threalose-6-phosphate phosphatase	0.45	0.83	0.89
TPS3.3	CA5505	Trehalose-phosphate synthase, regulatory subunit	0.41	0.49	0.92
IPF8321	CA2938	Initiator of glycogen synthesis	0.41	0.53	1.01
GSY1	CA5467	Glycogen synthase	0.45	0.51	1.04
GPH1	CA5206	Glycogen phosphorylase	0.35	0.57	0.98
RHR2	CA5788	DL-glycerol phosphatase	0.23	0.60	1.16
Citric acid cycle					
ACC1	CA5816	Acetyl-coenzyme-A carboxylase	1.5	1.42	1.08
ACH1	CA0345	Acetyl-coenzyme-A hydrolase	2.3	1.82	1.10
FUM12.3F	CA4351	Fumarate hydratase, 3-prime end	3.1	1.60	1.10
FUM12.5F	CA4349	Fumarate hydratase, 5-prime end	2.1	1.57	1.05
GDH3	CA1579	NADP-glutamate dehydrogenase	2.5	0.79	1.07
IPF4401	CA0903	Succinate dehydrogenase	1.7	0.99	1.24
KGD1	CA3149	2-oxoglutarate dehydrogenase	2	1.22	1.15
MDH1	CA5164	Mitochondrial malate dehydrogenase precursor	1.6	1.21	1.20
SDH12	CA2470	Succinate dehydrogenase	1.5	1.08	1.09
ACS2	CA2858	Acetyl-coenzyme-A synthetase	1.51	1.5	1.02

Expression of genes in an *efg1* mutant (HLC52), an *efh1* mutant (C4/d6-3) and an *efg1 efh1* mutant (H/1.22) relative to the wild-type CAF2-1. Cells were grown in YPD medium at 30°C prior to transcriptional profiling.

ADH5 and *MET6* were up-regulated by *EFG1* and *EFH1* overexpression (Supplemental Data, Figure 4). These experiments also revealed that in general, a different set of genes is regulated during the formation of true hyphae (dependent on Efg1p) compared with pseudohyphal formation (triggered by *EFG1* overexpression). Clearly, significant molecular differences exist between these morphological states.

Activity of Efg1p and Efh1p in One- and Two-Hybrid Assays

Efg1p is assumed by some to act as a transcriptional activator on the basis that the expression of hypha-specific genes depends upon Efg1p. However, other data suggest that Efg1 acts as a repressor. Therefore, we performed one- and two-hybrid assays to test directly whether the APSES proteins act as transcriptional activators or repressors.

In a first set of experiments, we used a recently developed one-hybrid assay, which allows an assessment of transcriptional regulatory activity in *C. albicans* (Russell and Brown, unpublished data). In these experiments LexA fusions to Efg1p or Efh1p were tested for their ability to repress or activate a *lacZ* reporter gene. This reporter was transcribed by a basal *ADH1* promoter fused to a *lexA* operator (Figure 9A, top). Transformants expressing *lexA* alone showed a basal level of β-galactosidase activity as indicated by blue staining on X-gal plates (Figure 9A, bottom). In contrast, β-galactosidase activity was significantly reduced in cells producing a LexA-Efg1 fusion. This reduction did not occur in a reporter lacking the *lexA* operator. Hence, Efg1p seems to act as a transcriptional repressor in *C. albicans*. Further experiments showed that the repressing function of the LexA-Efg1p was not affected by hypha-inducing stimuli, such as 10% serum or growth in microaerophilic conditions. In contrast to Efg1p, a LexA-Efh1p fusion significantly activated *lacZ* expression (Figure 9A, bottom), suggesting that Efh1 acts as a transcriptional activator.

In a second set of experiments, we performed standard two-hybrid assays in *S. cerevisiae* to examine the regulation and possible interactions of APSES proteins. For this purpose, we tested fusions of Efg1p or Efh1p to the Gal4p DNA-binding (BD) or activating domains (AD). In contrast to the one-hybrid assays in *C. albicans*, we found that BD-Efg1p-fusions did not influence *lacZ* reporter expression in *S. cerevisiae*. This suggests a requirement for *C. albicans*-specific components in the repressor function of Efg1p. In contrast, Efh1p-BD-fusions activated the *lacZ* reporter significantly (Figure 9B), which was consistent with the above-mentioned one-hybrid data obtained with *C. albicans.*

A C-terminally truncated Efh1p comprising 432 residues (N1) also activated the reporter gene in *S. cerevisiae*. However, further truncated versions retaining 275 or 159 residues (N2 and N3) were inactive (Figure 9B). These results suggested that Efh1p contains an activation domain between residues 275 and 432. An AD-Efh1p fusion did not activate the reporter, presumably because binding of the fusion protein to the promoter did not occur. How-

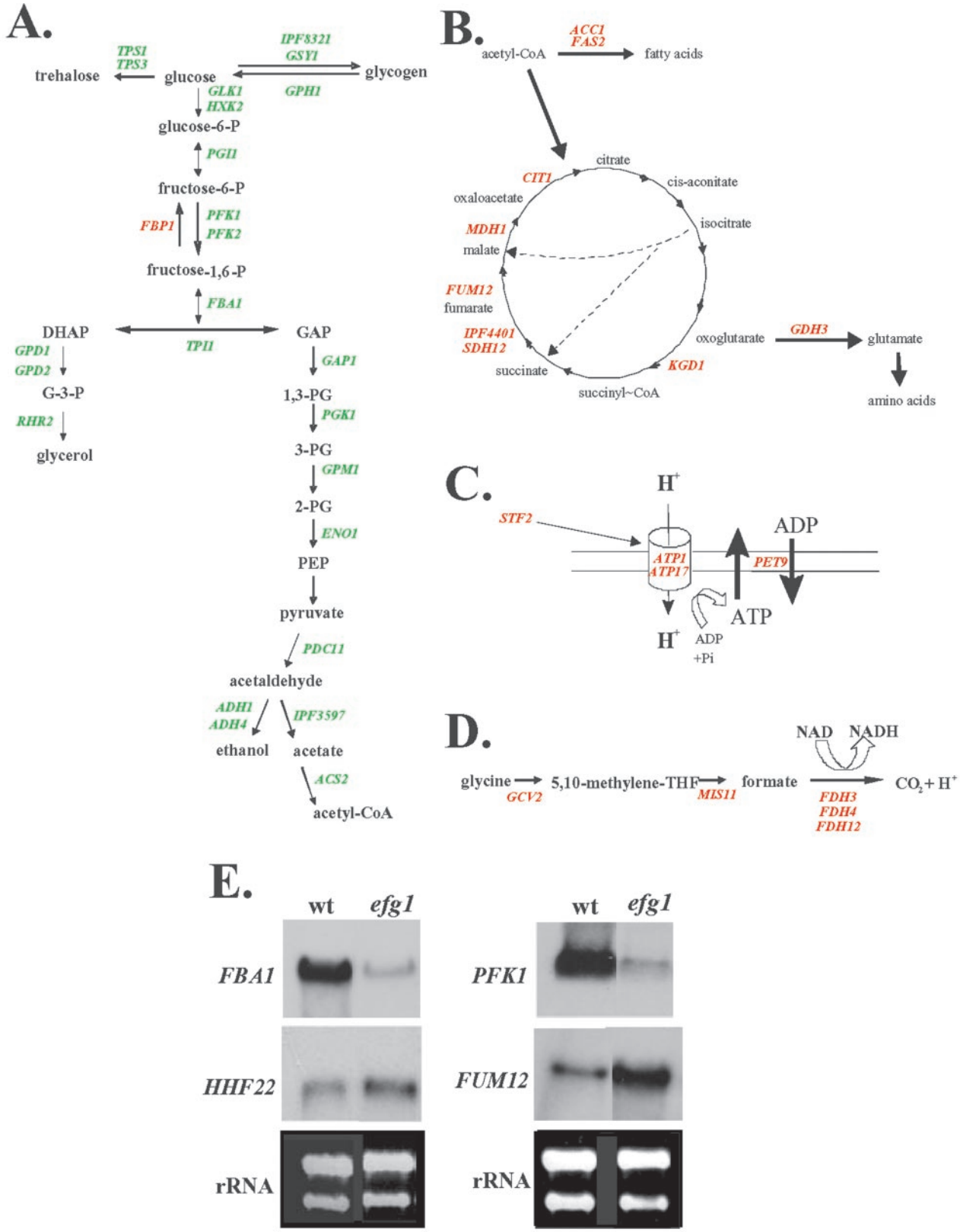

Figure 6. Regulation of genes involved in carbon metabolism by Efg1p. Genes regulated by Efg1p are involved in glycolysis (A), citric acid cycle (B), ATP biosynthesis (C), and glycin degradation (D) (genes up-regulated by Efg1p, green; genes down-regulated by Efg1p, red). (E) Northern blots on 30 μg of RNA of strain CAF2-1 (wt) and strain HLC52 (*efg1*), by using probes for the indicated genes. Equal loading was verified by ethidium-bromide staining of rRNA (rRNA).

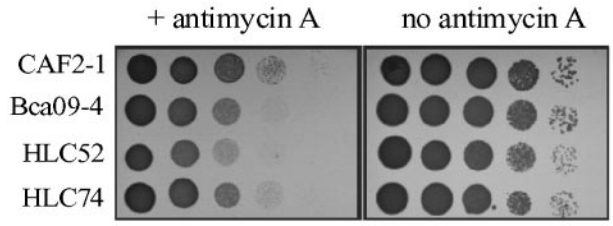

Figure 7. Supersensitivity of *efg1* mutants to antimycin A. Wild-type strain CAF2-1 and *efg1* mutants HLC52 and Bca09-4 (*efg1*), as well as an *efg1* mutant reconstituted with *EFG1* (HLC74) were grown in YPD and a series of tenfold dilutions was spotted on YPD agar containing 10 μg/ml antimycin A and grown at 30°C for 3 d.

ever, a strain containing both the BD-Efh1p fusion and the AD-Efh1p fusion contained twice the activity compared with a strain only containing a BD-Efh1p, suggesting homodimerization of Efh1p. This effect also was observed for the N1- and N2-proteins, but not the N3-truncated forms suggesting that N2 proteins are still able to dimerize and that a putative dimerization domain may be located between residues 159 and 275 of Efh1p. Similar

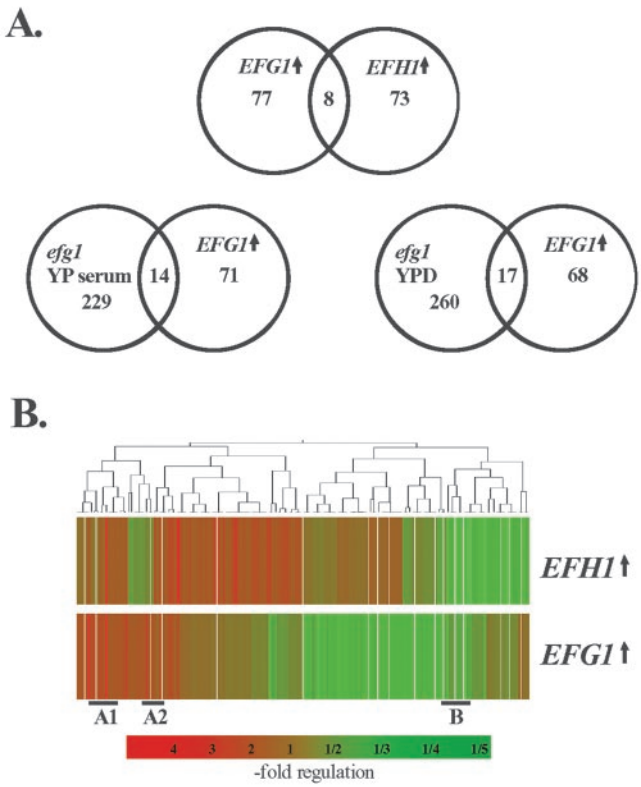

Figure 8. Genes regulated by *EFG1* or *EFH1* overexpression. (A) Venn diagram of 129 genes regulated by *EFG1*- (*EFG*↑) or *EFH1* (*EFH*↑) overexpression. Expression ratios of genes regulated in strain CAI4[pRC2312P-H] overexpressing *EFG1* or in strain CAI4[pDB35] overexpressing *EFH1* relative to a control transformant (CAI4[pBI-1]) grown identically were determined. Strains were grown in SCAA medium at 30°C. (B) Clustering of genes regulated by *EFG1/EFH1* overexpression. Genes in sections designated A1, A2, and B are shown in more detail in Supplemental Data, Figure 4.

experiments for the Efg1 protein did not reveal a homodimerizing activity for this transcriptional regulator nor heterodimerization with Efh1p.

From these experiments, we conclude that Efg1p and Efh1p have different regulatory potential. Efh1p functions as a transcriptional activator, as demonstrated in both *C. albicans* and *S. cerevisiae*. In contrast, Efg1p functions as a repressor, a function that for unknown reasons is only detected in the homologous host *C. albicans*. Furthermore, Efh1p but not Efg1p is able to form homodimers. As expected, the activation and dimerization domains of Efh1p are in regions that lack significant homology between these APSES proteins.

DISCUSSION

The availability of the complete *C. albicans* genomic sequence has allowed us to identify the complete set of APSES proteins in this organism, consisting of Efg1p and Efh1p. As a result, we were able to perform a comprehensive phenotypic and transcriptional analysis of APSES proteins, and in particular we were able to assess the relative contributions of both proteins to growth and morphogenesis. Furthermore, we examined functional interactions between both proteins and their cellular roles in *C. albicans*. This has allowed us to compare their functions to the APSES proteins of *S. cerevisiae*, which represent the only other known complete set of APSES protein in a fungal species.

Overexpression of both *EFG1* and *EFH1* induces pseudohyphal growth and represses the formation of true hyphae (Stoldt *et al*., 1997; Tebarth *et al*., 2003). At normal expression levels, both proteins cooperate to repress an alternative pathway of true hypha formation, which only seems to be operative in embedded or microaerophilic conditions. The function of the alternative pathway of filamentation in *C. albicans* is yet unknown, but it could promote morphogenesis in deep tissues or in phagocytic vacuoles when oxygen becomes limiting and/or cells are surrounded by an extracellular matrix. Our data suggest that the APSES proteins down-regulate this alternative morphogenetic pathway, thereby preventing filamentation under these conditions. On the other hand Efg1p is positively required for maintenance of the yeast growth form. In the absence of Efg1, cells change to a rod-like (*opaque*-like) form (Sonneborn *et al*., 1999b; Srikantha *et al*., 2000). Furthermore, Efg1p is required for the competence of yeast cells to undergo hyphal morphogenesis in standard (aerobic) conditions (Stoldt *et al*., 1997; Lo *et al*., 1997). Thus, Efg1p may have both activator and repressor functions in *C. albicans*, unlike APSES proteins in *S. cerevisiae*. Our one- and two-hybrid analyses confirmed a repressor function for Efg1p and an activator function for Efh1p, which had the ability to form homodimers in these tests. As expected, activation and homodimerization domains of Efh1p were mapped to regions outside of the conserved APSES domain.

Sequence comparisons reveal that Efh1p is more similar to the activator Phd1p, whereas Efg1p is more similar to the repressor Sok2p. For example, a potential PKA phosphorylation site is present in the APSES domains of Efg1p and Sok2 (RVT), but it is absent in Efh1p and Phd1 (KVA^{448} and RVI, respectively) (Bockmühl and Ernst, 2001). A direct role of Efg1p as a repressor was shown recently by its role as a negative autoregulator at the *EFG1* promoter (Tebarth *et al*., 2003). The apparent repressor function of Efh1p in embedded/hypoxic conditions may in fact reflect Efh1p-dependent activation of Efg1p repressor activity, or of *EFG1* expression under these conditions. Such an indirect role for Efh1p is

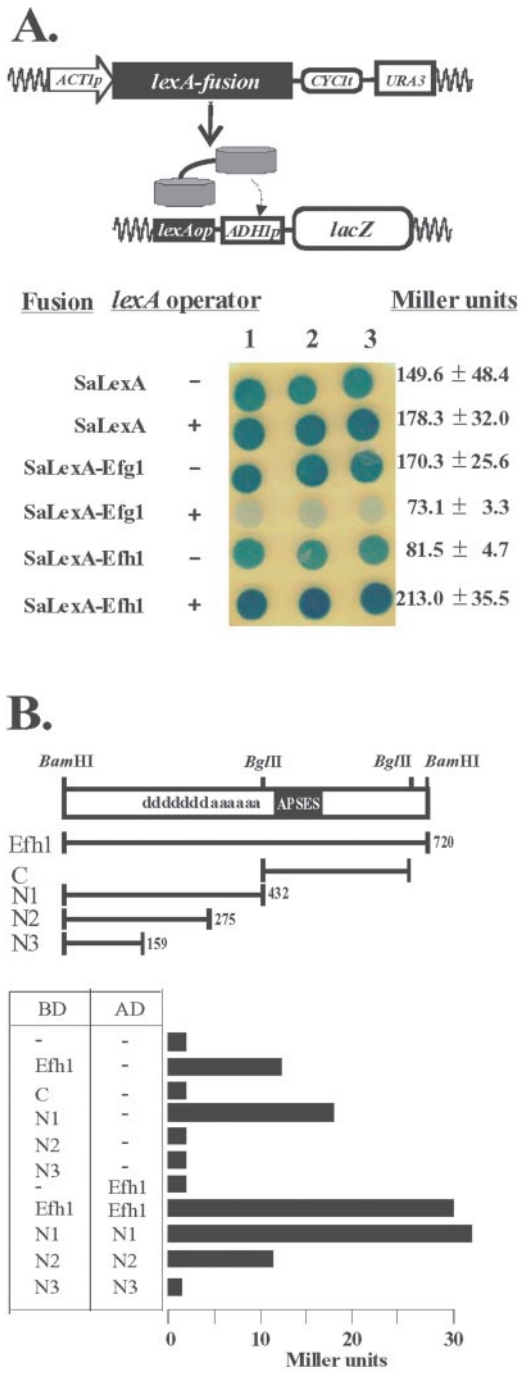

Figure 9. Transcriptional activities of Efg1p and Efh1p in one- or two-hybrid analyses. (A) One-hybrid assay in *C. albicans*. Expression plasmids encoding *S. aureus* LexA (SaLexA) or LexA-fusions to Efg1p (SaLexA-Efg1) or Efh1p (SaLexA-Efh1) were transformed into strain CRC104 (105, 106) carrying a *lacZ* reporter gene under control of a *lexA* operator (+). As controls strains CRC101 (102, 103), which carry *lacZ* without a *lexA* operator (−), were used as hosts. LacZ activity of cells grown on SD medium was determined using X-Gal indicator. (B) Two-hybrid assay in *S. cerevisiae*. Genes encoding full-length and truncated versions of Efh1p were inserted into pGBD- or GAD-vectors to express fusions to the Gal4p-DNA binding (BD) or activation domain (AD). Pairs of BD- or AD-plasmids were transformed into *S. cerevisiae* PJ69-4A; β-galactosidase levels of transformants, encoded by a *GAL7-lacZ* fusion in this strain, were determined in a liquid assay and expressed as Miller units. The localization of the APSES-domain in Efh1p, as well as presumed activation (a) and dimerization (d) domains, are indicated.

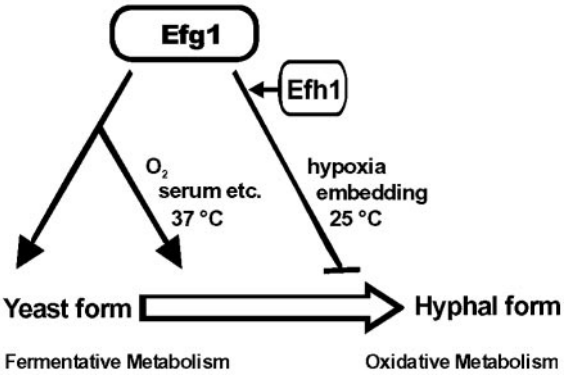

Figure 10. Model of APSES protein functions in *C. albicans*. Efg1p favors the yeast growth form and a fermentative mode of metabolism. It also is required for the transition from the yeast to the hyphal growth form, which is initiated by inducers including serum. In embedded and hypoxic conditions, at lower temperatures, Efg1p represses rather than induces hypha formation. Efg1p could act directly, as a direct transcriptional activator, or indirectly, by repressing yet undefined repressors of morphogenesis and metabolism (as suggested by Efg1p-repressor function in one-hybrid experiments). Efh1p enhances the activation and repression functions of Efg1p in metabolism and morphogenesis, presumably by activation of *EFG1* expression; Efh1p may also have yet unknown Efg1p-independent functions.

also suggested by the findings that *EFH1* overexpression phenotypes including pseudohypha- and *opaque-white*-induction, require Efg1p, and by the synthetic effects of Efh1 and Efg1 upon the *C. albicans* transcriptome. Thus, our data suggest that in *C. albicans*, the relative importance of the APSES proteins is skewed toward the main regulator Efg1p, whose role is facilitated under some conditions by the auxiliary factor Efh1p. In contrast, the *S. cerevisiae* APSES proteins seem to have opposing functions. A schematic overview of APSES protein functions in *C. albicans* is shown in Figure 10.

The influence of an *efg1* mutation upon expression of a limited set of genes, and more recently upon the *C. albicans* transcriptome, has been described previously (Lane *et al*., 2001; Nantel *et al*., 2002; Sohn *et al*., 2003). Our genome-wide transcript profiling extends these results significantly by 1) including the second APSES-encoding gene, *EFH1*; 2) examining the effects of overexpressing the APSES proteins; 3) analyzing the importance of APSES proteins at an early stage of hypha induction; and 4) establishing the role of APSES proteins in metabolism in addition to morphogenesis. Nantel *et al.* (2002) mainly described differences between the transcript profiles of cells under noninducing and inducing conditions. In contrast, we have compared mutant and wild-type cells under the same (inducing or noninducing) medium to identify even relatively small but statistically significant changes in the transcript profiles mediated by the APSES proteins. Also, we used strain CAF2-1 as the wild-type strain, because it is more directly related to the genetic background of the mutants we examined.

The inactivation of Efg1p is known to exert strong phenotypic effects upon *C. albicans* (Lo *et al*., 1997; Stoldt *et al*., 1997). Hence, it is not surprising that transcript profiling revealed that numerous genes were affected by the absence of Efg1p. In contrast, deletion of *EFH1* led to relatively few changes in the transcriptional profile. However, this was consistent with the minimal phenotypic effects of the *efh1* deletion.

When the functions of Efg1-regulated genes were examined, almost every gene encoding an enzyme involved in glycolysis was found to be stimulated by Efg1p. In contrast, genes encoding oxidative enzymes of the citric acid cycle were repressed by Efg1p. These differences were due to the loss of Efg1 rather than growth effects, because we examined the transcriptomes of *efg1* and wild-type cells under identical growth conditions. Thus, the presence of Efg1p favors fermentative and represses oxidative metabolism. A similar statement was made recently after comparisons of the transcriptomes of *white* and *opaque* cells. Lan *et al.* (2002) showed that the *white* (regular yeast) cells had enhanced expression of glycolytic genes compared with the *opaque* (rod-like) cells, which seemed to be in an state favoring oxidative metabolism. Intriguingly, *EFG1* expression is insignificant in *opaque* cells, and *efg1* mutants have an *opaque*-like appearance (Sonneborn *et al*., 1999b). Therefore, the overlaps between the transcriptomes of *opaque* and *efg1* cells might be due, at least in part, to the absence of Efg1p. We show that the effects of Efg1p disruption upon glycolysis are of biological significance, because the growth of *efg1* mutants is relatively sensitive to antimycin A, an inhibitor of respiration.

EFG1 and *EFH1* overexpression affected the expression of several genes in common. This was to be expected because the overexpression of both genes causes similar phenotypes, including the induction of pseudohyphal growth and promotion of *opaque* to *white* phenotypic switching. The induction of pseudohyphal growth by *EFH1* required the presence of *EFG1*, suggesting that Efh1p acts indirectly to stimulate *EFG1* expression or Efg1p activity, which consequently would lead to (partially) overlapping transcriptional profiles. Genes commonly regulated by Efg1p- and Efh1p-overproduction included *ALS1*, *ALS10*, and *HWP1*. This is probably closely correlated to the pseudohyphal or filamentous growth modes of these cell types (Braun and Johnson, 2000; Lane *et al*., 2001). Nevertheless, other genes were differentially regulated by Efg1p and Efh1p, probably reflecting the structural differences of both proteins. Interestingly, the set of genes identified in overexpression experiments did not overlap significantly with genes affected by the absence of APSES proteins in hypha-inducing conditions. Again, this highlights that significant differences exist between pseudohyphal and true hyphal modes of growth. Surprisingly, overexpression of *EFG1* and *EFH1* down-regulated *IPF5185* (*FLO1*), encoding a putative yeast-specific cell wall protein, although in an *efg1* mutant it was down-regulated as well (Nantel *et al*., 2002; Sohn *et al*., 2003). This can be explained by the suggestion that Efg1p acts as an activator as well as a repressor for this gene (Sohn *et al*., 2003).

To summarize, the results presented in this study suggest that APSES proteins are important regulators of metabolism and morphogenesis in *C. albicans*. Their effects upon morphogenesis include inducible dimorphism and spontaneous phenotypic switching. The typical yeast form (*white*) exploits a fermentative mode of metabolism, which is enhanced by the APSES protein Efg1p. This also renders these cells competent to form true hyphae in standard inducing conditions (lack of glucose, presence of inducers, 37°C). Homozygosity at the *MTL* locus, low temperatures (Miller and Johnson, 2002), or the down-regulation of *EFG1/EFH1* (Sonneborn *et al*., 1999b) allows the generation of the *opaque* form, which exploits an oxidative mode of metabolism. This is refractory to standard inducers of hyphal growth.

We previously pointed out several structural and functional parallels between Efg1p and human myc proteins (Stoldt *et al*., 1997; Tebarth *et al*., 2003). Interestingly, it has been reported recently that, in addition to its role in cellular differentiation, myc induces fermentative metabolism (in particular glucose import and glycolysis: Osthus *et al*., 2000). Intriguingly, Myc-like proteins in *Drosophila* bind to ~15% of all coding regions (Orian *et al*., 2003) suggesting a general role in gene expression. Collectively, the data suggest that APSES proteins play important roles in coordinating central metabolism with cellular differentiation.

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