

Construction and real-time RT-PCR validation of *Candida albicans* PALS-GFP reporter strains and their use in flow cytometry analysis of ALS gene expression in budding and filamenting cells

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The gene encoding yeast-enhanced green fluorescent protein (GFP) was placed under control of ALS gene promoters in *Candida albicans*. The PALS-GFP reporter strains were validated using various techniques including a new real-time RT-PCR assay to quantify ALS gene expression. The PALS-GFP reporter strains were grown in media that promoted yeast or germ tube forms, and the resulting fluorescence was measured by flow cytometry. In addition to results that indicate differences in ALS gene expression due to growth medium, growth stage and developmental programme, new data show large differences in transcriptional level among the ALS genes. Expression of *ALS1* was associated with transfer of the *PALS1*-GFP strain to fresh growth medium. *ALS3* expression increased markedly when germ tubes were visible microscopically and *ALS7* expression exhibited a transient peak between 2 and 3 h following inoculation into fresh YPD medium. Transcription from the *ALS1* and *ALS3* promoters was strongest among those tested and contrasted markedly with the weaker promoter strength at the *ALS5*, *ALS6*, *ALS7* and *ALS9* loci. These weaker transcriptional responses were also observed using real-time RT-PCR measurements on wild-type *C. albicans* cells. Assuming a positive correlation between transcriptional level and protein production, these results suggest that some Als proteins are abundant on the *C. albicans* cell surface while others are produced at a much lower level.

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INTRODUCTION

Although *in vivo* disease models are used frequently to study microbial pathogens, many experiments to characterize pathogenesis-related genes are conducted *in vitro*. The pathogenic fungus *Candida albicans* has numerous genes that contribute to disease processes, and some of these are organized into gene families (Jones *et al.*, 2004). One example is the ALS (agglutinin-like sequence) gene family that encodes cell-surface glycoproteins involved in adhesion to host surfaces and potentially other cellular processes (Gaur & Klotz, 1997; Hoyer, 2001; Fu *et al.*, 2002; Zhao *et al.*, 2004; Sheppard *et al.*, 2004). *In vitro* regulation of the ALS genes has been studied using various methodologies. ALS genes were described as differentially regulated when studied by Northern blotting. *ALS1* expression was dependent on growth conditions (Hoyer *et al.*, 1995), *ALS3* was transcribed only in germ tubes and hyphae (Hoyer *et al.*, 1998a) and *ALS4* was up-regulated strongly in cells from overnight cultures (Hoyer *et al.*, 1998b). Since transcripts specific for some of the other ALS genes were more difficult

to detect by Northern blotting of RNA extracted from cultured cells, specific primers for RT-PCR analysis were developed (Green *et al.*, 2004). The increased sensitivity of this technique revealed that transcripts for all ALS genes could be detected under many different experimental conditions, including inoculation into the RHE disease model and following growth in model biofilms (Green *et al.*, 2004). Although sensitive, the RT-PCR technique is laborious since it requires extraction of RNA, DNase treatment and rather extensive experimental controls. In addition, the original set of RT-PCR primers were not suitable for quantification of ALS transcript abundance using real-time techniques since the PCR products were too large for optimal analysis.

Another approach to studying ALS gene expression *in vitro* involves the construction of reporter strains. This manuscript describes use of green fluorescent protein (GFP) as a reporter of ALS promoter activity and flow-cytometry measurement of the resulting fluorescent signal in cultured *C. albicans* cells. Fusions of GFP to inducible promoters in *C. albicans* showed rapid production of GFP under inducing conditions and quick protein decay under repressing conditions, and demonstrated its effectiveness as a reporter

Abbreviations: GFP, green fluorescent protein; GMF, geometric mean fluorescence.

of gene regulation (Barelle *et al.*, 2004). Gene expression patterns of the PALS-GFP strains described here are verified against those in wild-type *C. albicans* using a newly developed real-time RT-PCR assay for the ALS family. The PALS-GFP clones provide a dynamic analysis of ALS gene expression that is quick and inexpensive compared to RT-PCR methods. This method provides a quantitative evaluation of fluorescence for comparison of the strength of the various ALS promoters. Culture conditions examined include those used to study ALS gene expression in previous work. Data derived from these studies show that some ALS genes are regulated by large increases in transcription while others have more low-level and subtle regulatory changes. Data presented here also identify *in vitro* conditions that can be used for phenotypic analysis of *als/als* mutant strains.

METHODS

Culture media. Three different media were used for *C. albicans* cultures. YPD medium consisted of 1% yeast extract, 2% peptone and 2% glucose and was sterilized by autoclaving. YNB+Glc medium consisted of 6.7 g Difco Yeast Nitrogen Base (catalogue no. 239210) and 9 g glucose per litre and was sterilized by filtration. RPMI 1640 medium without L-glutamine (RPMI; Invitrogen, catalogue no. 11875-085) or YPD with 10% (v/v) fetal calf serum (Invitrogen) was used for growing germ tubes.

Construction of *C. albicans* PALS-GFP reporter strains. All experiments used yeast-enhanced GFP (Cormack *et al.*, 1997). GFP reporter constructs targeted for integration into specific ALS loci were generated using the method of Gerami-Nejad *et al.* (2001). Plasmid pGFP-URA3 was a generous gift from Cheryl Gale (University of Minnesota, Minneapolis, MN, USA). Plasmid pGFP-URA3 contains a reporter cassette with the GFP gene and the URA3 selectable marker, and was used as the PCR template to generate integration cassettes. General guidelines were used to design each set of PCR amplification primers for the eight ALS genes (Table 1). Forward primers included 57 nt of sequence upstream of the ALS gene, followed by an ATG and 20 nt of plasmid pGFP-URA3 sequence (5'-ATG TCT AAA GGT GAA GAA TTA TT-3'). Reverse primers included 57 nt downstream of the ALS gene and 23 nt of plasmid pGFP-URA3 sequence (5'-TCT AGA AGG ACC ACC TTT GAT TG-3'). Each cassette was designed so that it could be 'knocked into' the correct ALS locus, thereby generating ALS heterozygotes where one ALS allele was replaced with the GFP-URA3 cassette, while maintaining the native promoter sequence. PCR products were amplified using Taq polymerase (Invitrogen) and transformed into *C. albicans* CAI4 (Fonzi & Irwin, 1993) using previously described methods (Zhao *et al.*, 2004). CAI4 was a generous gift from William Fonzi (Georgetown University, Washington, DC, USA). Transformants were selected on synthetic complete medium without uridine (SC-Uri; Hicks & Herskowitz, 1976) containing 1 M sorbitol.

Southern blot and culture-based validation of *C. albicans* PALS-GFP reporter strains. Transformants were grown in liquid SC-Uri for DNA extraction. Genomic DNA was extracted from *C. albicans* using the MasterPure Yeast DNA Purification Kit (Epicentre). Transformant DNA was digested with the appropriate restriction enzyme (Table 2), and screened by Southern blotting using Genius reagents and chemiluminescent detection (Roche). Methods for construction of the Southern blot probes are outlined below. In order to maintain as many of the wild-type *C. albicans* pathogenesis attributes as possible, transformants in which the GFP

cassette had disrupted the smaller ALS allele were chosen. This decision was made based on previous studies that demonstrated a difference in adhesion that was dependent on allele length, with smaller alleles conferring less adherent phenotypes (Oh *et al.*, 2005). It was therefore assumed that replacement of the small allele of a given ALS gene would not result in haploinsufficiency at that locus or otherwise interfere with cell growth or virulence. Growth rates of the *C. albicans* strains constructed in this study were assessed in YPD liquid medium as described by Zhao *et al.* (2004) and matched to the growth rate of strain CAI12 (Porta *et al.*, 1999), which is a URA3 derivative of CAI4. Strain CAI12 was provided by William Fonzi (Georgetown University). Integration of the GFP reporter cassette was particularly difficult at the ALS2 locus. Screening of over 800 transformants yielded only one with the appropriate restriction pattern on a Southern blot. Since this clone had a significantly slower growth rate than the remainder of the reporter strains, its analysis was omitted from this publication. Because the ALS2 and ALS4 loci share very similar sequences, analysis of the PALS4-GFP reporter strain will be presented in a separate paper with the PALS2-GFP data when an appropriate ALS2 reporter clone is isolated.

Southern blot probe construction. The GFP coding region was amplified for use as a Southern blot probe with primers GFPXhoI and GFPBglII (Table 1). The remaining Southern blot probes were PCR-amplified sequences from upstream of each ALS gene. Forward primers were designed to contain a *KpnI* restriction site, and reverse primers contained a terminal *XhoI* site. Promoter fragments for cloning were amplified with *Pfu* polymerase (Stratagene) to ensure sequence fidelity. PCR products were purified by phenol/chloroform extraction and ethanol precipitation, then digested with *KpnI/XhoI*. Digestion products were resolved on 0.7% agarose gels, and purified using GeneClean (Qiagen), cloned into CIP10 (Murad *et al.*, 2000), and transformed into *Escherichia coli*. Plasmid constructs were recovered from *E. coli* and DNA sequenced to verify the identity of the fragment. Probe fragments were excised, purified and labelled using Genius reagents (Roche) for use in Southern blotting.

RT-PCR analysis of GFP expression in PALS-GFP strains. The PALS-GFP reporter strains were monitored under *in vitro* conditions in order to correlate GFP mRNA expression of each reporter strain with published RT-PCR data for the ALS family (Green *et al.*, 2004). RNA was isolated from 1 ml of 16 h YPD cultures using a hot phenol extraction method (Collart & Oliviero, 1993). RT-PCR was performed as previously described (Green *et al.*, 2004), but using GFP-specific primers GFPRTF and GFPRTR (Table 1).

Real-time RT-PCR analysis of ALS gene expression. Real-time RT-PCR primers were designed using PRIMEREXPRESS software (version 2.0; Applied Biosystems) to have a T_m between 59 and 60 °C and an amplicon size of 50–100 bp (Table 3). DNA sequencing of the PCR product amplified from *C. albicans* genomic DNA verified primer specificity. *C. albicans* CAI12 was grown as for flow cytometry analysis (see below) for 16 h in YPD or in RPMI for 30 min, both at 37 °C and 200 r.p.m. shaking. Total RNA was extracted (Collart & Oliviero, 1993) and further purified using RNeasy (Qiagen). RNA was treated with DNase as described previously (Green *et al.*, 2004) and purified again with RNeasy. cDNA was synthesized using the SuperScript First Strand Synthesis System (Invitrogen) with 1 µg RNA as the template. Following synthesis, cDNA was diluted 1:5 with sterile MilliQ water. PCR reactions contained 100 nM of each primer, 1 × Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen), 1 × ROX reference dye (Invitrogen) and 5 µl diluted cDNA in a final volume of 25 µl. PCR reactions were run on the ABI Prism 7000 Sequence Detection System (SDS) with a 2 min 50 °C UDG incubation step, and 95 °C incubation for 2 min, followed by 40 cycles of 95 °C (15 s) and 60 °C (1 min). All primer pairs produced a single amplicon with a uniform melting curve as determined by the dissociation profile of the product. A

Table 1. Oligonucleotide primers

Primer	Sequence (5'→3')*
ALS1-FPF	GTT TTT TCG TTT TAC TTC ATC AGA ATT GTT CAA ACA ACT ACC AAT TGT TAA TAT CAG ATG TCT AAA GGT GAA GAA TTA TT
ALS1-FPR	CGA AGA AAA GAT AAA TGT GAA CTA GAT CAA GCC AAA AAG GTG ATC ATA ACA ATA TAG <i>TCT</i> AGA <i>AGG ACC ACC TTT GAT TG</i>
ALS3-FPF	AGT TTT TAA TTT CAT TTT ATT ATA ATT GTA TAA ACA ACT ACC AAC TGC TAA TAT TAG ATG TCT AAA GGT GAA GAA TTA TT
ALS3-FPR	AAA CAA AAA ACA AAC AAA TAA CAA AAA TCT AAA AAG GCG ACT ATG ATG GTA TCA TCC <i>TCT</i> AGA <i>AGG ACC ACC TTT GAT TG</i>
ALS5-FPF	CAT TGT TAG TTT CAC TTC TTC AGA ATT GTT CAA ACA ACT ACC AAC TGC TAA CAC CAG ATG TCT AAA GGT GAA GAA TTA TT
ALS5-FPR	ATT TGG TAC AAT CCC GTT TGA ATA AAA ACA GAA CTA AAA AAA CGA CAC CAA AAA TGA <i>TCT</i> AGA <i>AGG ACC ACC TTT GAT TG</i>
ALS6-FPF	TAG GAA ATT TCA TTC ATT GAC TTG TAT AAA CAT CAT CAG CAT CAC CAT TCA CCG ACA ATG TCT AAA GGT GAA GAA TTA TT
ALS6-FPR	ATT TGG TAC AAT CCC GTT TGA ATA AAA ACA GAA CTA AAA AAA CGA CAC CAA AAA TGA <i>TCT</i> AGA <i>AGG ACC ACC TTT GAT TG</i>
ALS7-FPF	GAA AAT AAG AAT TTT TCA TCA ATC TAA CAA TCT ACA ATT TTC AAC AGT CTA ATA CCT ATG TCT AAA GGT GAA GAA TTA TT
ALS7-FPR	TGA AAA TTC ATA ACG AAA ATC TTG AAA AAA AAA ATA TAA ATA AAA TTT AAA AGT GAA <i>TCT</i> AGA <i>AGG ACC ACC TTT GAT TG</i>
ALS9-FPF	GTT TTG CAA TCT TTT CAT TTT TGT TGT TTG AAA ACC CTT AAT CAC CAA GAA TTT CAA ATG TCT AAA GGT GAA GAA TTA TT
ALS9-FPR	TAA CAT CAC GAA GAA ATA ATA AAA GGG TAT TCA AAC AAA CTT ATG ACA GTA TAG TTA <i>TCT</i> AGA <i>AGG ACC ACC TTT GAT TG</i>
GFPXhoI	CCC TCG AGT ATT AAA ATG TCT AAA GGT GAA GAA TTA TTC ACT
GFPBglII	CCC ACG CGT CTG CTT GTA GAG TTG ATA GGA ATC ATC
ALS1GFPP	CCC GGT ACC ATC ACT CTT CAA TAA TCC CAT ACT
ALS1GFPR	CCC CTC GAG CTG ATA TTA ACA ATT GGT AG
3upABKpn	CCG GTA CCA TAA TAA GAC AAA AAT AAA AAG
3upABXho	CCC CTC GAG CTA ATA TTA GCA GTT GGT AGT TG
ALS5F13	CCC GGT ACC CAA CTA AAA CTT TAT CAT CAA ATC AC
ALS5GFPR	CCC CTC GAG CTG GTG TTA GCA GTT GGT AGT TGT TTG
ALS6GFPP	CCC GGT ACC TGT TTC AAT CAA TTG CCT ATC
ALS6GFPR	CCC CTC GAG TGT CGG TGA ATG GTG ATG CTG
ALS7GFPP	CCC GGT ACC CCC AAT AAA TAA TAA TGA ACA CAA AAA
ALS7GFPR	CCC CTC GAG AGG TAT TAG ACT GTT GAA AAT TGT AGA T
ALS9GFPP	CCC GGT ACC TTT TTC TTT TCC TTG TTT TCC
ALS9GFPR	CCC CTC GAG TTG AAA TTC TTG GTG ATT AAG
GFPRTF	TCT GTC TCC GGT GAA GGT GAA G
GFPRTR	GGC ATG GCA GAC TTG AAA AAG

*Bold type indicates the ATG start codon within forward primers for amplification of the GFP cassette. Italicized type indicates sequence from the pGFP-URA3 plasmid within reverse primers for amplification of the GFP cassette.

standard curve was constructed for each primer set with 1:10, 1:25, 1:50, 1:100, 1:250 and 1:500 dilutions of cDNA. The slopes of the standard curves were within 10% of 100% efficiency. C_T values were determined using the AUTOANALYSE features of the SDS software.

Transcript copy number was calculated by running each primer pair with the corresponding cloned ALS or *TEF1* gene sequence. Plasmids were isolated, treated with RNase and purified using the Wizard DNA Clean-Up System (Promega). Plasmid concentrations were determined spectrophotometrically and copy numbers calculated based on plasmid

mass. A dilution series from 10^2 to 10^6 plasmid copies was used to generate a standard curve. Absolute quantification was used to determine the input transcript copy number per 0.05 μ g RNA, by relating the C_T value to the standard curve as demonstrated by Heid *et al.* (1996). Reactions to calculate transcript copy number were run in duplicate from two separate RNA preparations. Means and standard errors were calculated in SAS (SAS Institute).

Preparation of PALS-GFP strains for flow cytometry analysis. Frozen stocks of *C. albicans* strains were streaked onto YPD

Table 2. PALS-GFP reporter strain validation information

Reporter construct	PALS-GFP strain name	Enzyme digest	GFP-hybridizing Southern blot fragment size (kb)	Primers for upstream probe synthesis
PALS1-GFP	2225	<i>Bgl</i> III	1·4	ALS1GFPP ALS1GFPR
PALS3-GFP	2185	<i>Eco</i> RV	1·6	3upABKpn 3upABXho
PALS5-GFP	2227	<i>Bgl</i> III	11	ALS5F13 ALS5GFPR
PALS6-GFP	2223	<i>Bgl</i> III	8·0	ALS6GFPP ALS6GFPR
PALS7-GFP	2224	<i>Bgl</i> III	2·0	ALS7GFPP ALS7GFPR
PALS9-GFP	2337	<i>Bgl</i> III	2·3	ALS9GFPP ALS9GFPR

agar plates and incubated at 37 °C for 24 h. A single colony of each strain was resuspended in 1 ml sterile YPD liquid and vortexed vigorously to resuspend the cells. Twenty microlitres of this suspension were used to inoculate 10 ml fresh YPD liquid or YNB+Glc medium. Cultures were incubated for 16 h at 37 °C with 200 r.p.m. shaking. An aliquot of each starter culture was observed microscopically to ensure that only yeast forms were present. Cells were collected by centrifugation, washed twice in sterile PBS (per litre: 10 g NaCl; 0·25 g KCl; 1·43 g Na₂HPO₄; pH 7·2) and counted in duplicate. One hundred millilitres of fresh growth medium was inoculated at a density of 1×10^6 cells ml⁻¹ and incubated at 37 °C with 200 r.p.m. shaking. Every hour, an aliquot of each culture was removed and the cells were collected by centrifugation, washed twice in PBS and analysed by flow cytometry. An additional aliquot of

culture was fixed with glutaraldehyde (final concentration 1%) and used for triplicate optical density readings. OD₆₂₀ readings were taken in a microplate format using the iEMS Reader MF (Thermo Labsystems).

PALS-GFP reporter strains were also grown in RPMI medium and YPD with 10% serum for flow cytometry analysis. Starter cultures were prepared in YPD medium as described above. *C. albicans* cells were harvested by centrifugation, washed three times in PBS and counted in triplicate. Cells were resuspended in 20 ml RPMI or YPD with 10% serum at a concentration of 5×10^6 cells ml⁻¹ and incubated in a 37 °C water bath with 200 r.p.m. shaking. An aliquot of each culture was collected and analysed by flow cytometry every 15 min for 1 h. The percentage germ tube formation was determined for each

Table 3. Primers for real-time RT-PCR analysis of ALS gene expression

Gene	GenBank entry	Primers	Primer sequence (5'→3')	Seq. coord.	PCR product size (bp)
<i>ALS1</i>	L25902	QRTALS1F	TTC TCA TGA ATC AGC ATC CAC AA	3213	53
		QRTALS1R	CAG AAT TTT CAC CCA TAC TTG GTT TC	3265	
<i>ALS2</i>	AF024580	QRTALS2F	TTC CAA GTA TTA ACA AAG TTT CAA TCA CTT A	569	68
		QRTALS2R	ACC AGA TGT GTA GCC ATT TGC AC	636	
<i>ALS3</i>	U87956	QRTALS3F	AAT GGT CCT TAT GAA TCA CCA TCT ACT A	2857	56
		QRTALS3R	GAG TTT TCA TCC ATA CTT GAT TTC ACA T	2912	
<i>ALS4</i>	AF272027	QRTALS4F	TCT GCA ACA CGA GTC AGC TCA	841	64
		QRTALS4R	CCG CAC CAA CAC AAG CAT ATA T	904	
<i>ALS5</i>	AY227440	QRTALS5F	CTG CCG GTT ATC GTC CAT TTA	806	63
		QRTALS5R	ATT GAT ACT GGT TAT TAT CTG AGG GAG AAA	868	
<i>ALS6</i>	AF075293	QRTALS6F	GAC TCC ACA ATC ATC TAG TAG CTT GGT TT	528	52
		QRTALS6R	ACT TGG AAT AAC CCT TGC GAA A	579	
<i>ALS7</i>	AF201684	QRTALS7F	GAA GAG AAC TAG CGT TTG GTC TAG TTG T	530	51
		QRTALS7R	GCG ACA TGG AAA GTC TTT GAC TAA C	580	
<i>ALS9</i>	AY269423	QRTALS9F	AAA TCA ATT ACC ACC CCA GCT G	4849	68
		QRTALS9R	GAA ACT GAA ACT GCT GGA TTT GG	4916	
<i>TEF1</i>	orf19.5119*	QRTTEF1F	CCA CTG AAG TCA AGT CCG TTG A	851	51
		QRTTEF1R	CAC CTT CAG CCA ATT GTT CGT	901	

*The *TEF1* gene sequence was obtained from the genome annotation data at <http://candida.bri.nrc.ca/candida>.

replicate at 1 h. The reporter strains and CAI12 exhibited 90–99% germ tubes with length greater than or equal to the diameter of the mother yeast, suggesting that disruption of the small allele of each ALS gene did not result in altered filamentation in RPMI (data not shown).

Flow cytometry. Flow cytometry was performed using a Beckman Coulter EPICS XL machine. This instrument is equipped with a 15 mW air-cooled argon laser with an excitation wavelength of 488 nm. For fluorescence analysis, a region was set on a histogram, which represented side-angle light scatter versus forward-angle light scatter for a population of CAI12 yeast-form cells. The fluorescence was then gated on this region. Ten thousand events were collected at the medium flow rate. Fluorescence was measured on the FL1 fluorescence channel equipped with a 525 nm emission bandpass filter. Geometric mean fluorescence values for each time point were calculated using WINLIST software (Verity).

RESULTS

Validation of PALS-GFP reporter constructs

Reporter constructs were validated by Southern blotting using different probes as described in Methods. Southern blots of genomic DNA from the different PALS-GFP reporter strains were hybridized with the GFP coding region probe and showed fragment sizes corresponding to those predicted from DNA sequence information (Table 2), suggesting that the correct set of PALS-GFP constructs was obtained. Additional Southern blots with probes that hybridized upstream of each ALS coding region confirmed these results (data not shown).

PALS-GFP reporter constructs were also verified by RT-PCR analysis. Previous work showed that expression of all eight ALS genes could be detected by RT-PCR using ALS-specific primers and RNA extracted from cells grown overnight at 37 °C in YPD medium (Green *et al.*, 2004). RT-PCR analysis was conducted using GFP-specific primers GFPRTF and GFPRTR (Table 1) and RNA extracted from a YPD culture of each PALS-GFP reporter strain grown overnight at 37 °C. Strain CAI12, which does not encode GFP sequences, was included as a negative control. Amplification of RNA without reverse transcription was used as a further negative control to ensure that the signals obtained resulted from the cDNA rather than from contaminating genomic DNA. All DNase-treated RNA preparations produced negative results (data not shown). RT-PCR products of the predicted size for the GFP primers indicated that GFP was transcribed from each ALS locus in a manner similar to that observed in the wild-type parent strain (Fig. 1; Green *et al.*, 2004). Real-time RT-PCR was also used to verify that GFP production in the reporter strains matched ALS gene expression in CAI12; these results are presented with the flow cytometry data below.

Flow cytometry analysis of *C. albicans* PALS-GFP yeast forms

The collection of PALS-GFP strains was grown in YPD medium as described in Methods and the geometric mean

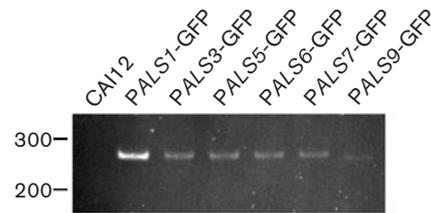


Fig. 1. Ethidium bromide-stained acrylamide gel of RT-PCR products amplified with GFP-specific primers. Total RNA was extracted from strain CAI12 and the PALS-GFP reporter strains grown for 16 h in YPD at 37 °C. RNA samples were DNase treated and shown to be negative for genomic DNA. PCR with GFP-specific primers provided evidence of transcription of each ALS gene under this growth condition, consistent with previous studies (Green *et al.*, 2004). Molecular size markers (bp) are shown on the left.

fluorescence (GMF) calculated from flow cytometry measurements at 1 h time points (Table 4). The strains were also examined during growth in YNB+Glc medium (Table 5). Both of these growth conditions are relevant to the analysis of ALS gene expression since the ALS family was first discovered following growth in YPD (Hoyer *et al.*, 1995), and YNB+Glc medium is used for biofilm growth in the denture and catheter models (Chandra *et al.*, 2001; Kuhn *et al.*, 2002). Growth in two different conditions also allowed for the comparison of results in order to dissect the effects of growth stage compared to growth medium. Optical density values were recorded to describe the stage of growth for each culture. Strain CAI12 was included in each analysis to account for the background autofluorescence of *C. albicans* cells (Mann, 1983).

Expression from the *ALS1* locus was strongly influenced by growth stage, with GFP production increasing rapidly and to high levels upon inoculation of cells from an overnight culture into fresh medium. As the growth of this new culture progressed, fluorescence readings steadily dropped to approximately the level of the background culture at 16 h (Table 4). GFP production by the PALS3-GFP strain was similar to background levels in these yeast-form cultures, consistent with previous evidence that *ALS3* is transcribed in germ tubes and hyphae (Hoyer *et al.*, 1998a). Fluorescence readings were consistently higher than the control for the PALS5-GFP strain, suggesting a low level of transcription across the growth curve. A similar pattern of GFP readings was observed for the PALS6-GFP strain, with the highest relative increase over CAI12 control readings found later in the growth curve. The highest relative increase over control readings for the PALS7-GFP strain occurred between 2 and 3 h of growth. Additional experimentation to further verify this transient fluorescence increase showed that it was repeatable and statistically significant ($P < 0.0001$; Table 6). GMF readings for the PALS9-GFP strain were similar to control values, suggesting weak levels of transcription from

Table 4. GMF and OD₆₂₀ readings for yeast forms of the PALS-GFP strains grown in YPD medium

Fluorescence intensity of budding cells was measured every hour with a Beckman Coulter EPICS XL machine. Corresponding OD₆₂₀ readings were taken for each culture, at each time point, in order to associate fluorescence with the stage of growth. OD and GMF are reported in arbitrary units. Values are the mean \pm SEM taken from three separate experiments.

Time (h)	CAI12		PALS1-GFP		PALS3-GFP		PALS5-GFP		PALS6-GFP		PALS7-GFP		PALS9-GFP	
	OD	GMF												
1	0.1 \pm 0.0	2.6 \pm 0.4	0.1 \pm 0.0	68 \pm 2.7	0.1 \pm 0.0	2.4 \pm 0.1	0.1 \pm 0.0	2.7 \pm 0.2	0.2 \pm 0.0	2.8 \pm 0.1	0.2 \pm 0.0	2.5 \pm 0.4	0.2 \pm 0.0	2.2 \pm 0.1
2	0.2 \pm 0.0	2.9 \pm 0.3	0.1 \pm 0.0	70 \pm 8.0	0.1 \pm 0.0	2.7 \pm 0.2	0.1 \pm 0.0	3.4 \pm 0.2	0.2 \pm 0.0	3.0 \pm 0.4	0.2 \pm 0.0	3.9 \pm 0.3	0.2 \pm 0.0	2.8 \pm 0.2
3	0.2 \pm 0.0	2.2 \pm 0.0	0.2 \pm 0.0	27 \pm 11	0.1 \pm 0.0	2.3 \pm 0.2	0.1 \pm 0.0	2.9 \pm 0.0	0.2 \pm 0.0	2.3 \pm 0.1	0.2 \pm 0.0	3.7 \pm 0.6	0.2 \pm 0.0	2.1 \pm 0.3
4	0.3 \pm 0.0	1.9 \pm 0.0	0.2 \pm 0.0	15 \pm 0.3	0.2 \pm 0.0	1.9 \pm 0.1	0.2 \pm 0.0	2.5 \pm 0.2	0.3 \pm 0.0	2.2 \pm 0.2	0.2 \pm 0.0	2.9 \pm 0.2	0.2 \pm 0.0	2.0 \pm 0.1
5	0.4 \pm 0.0	1.8 \pm 0.1	0.3 \pm 0.0	8.2 \pm 1.1	0.3 \pm 0.0	1.8 \pm 0.1	0.2 \pm 0.0	2.3 \pm 0.1	0.4 \pm 0.0	2.3 \pm 0.0	0.4 \pm 0.1	2.4 \pm 0.1	0.3 \pm 0.0	1.8 \pm 0.1
6	0.7 \pm 0.0	1.6 \pm 0.0	0.4 \pm 0.0	4.4 \pm 0.4	0.4 \pm 0.0	1.9 \pm 0.0	0.4 \pm 0.0	2.5 \pm 0.1	0.7 \pm 0.0	1.8 \pm 0.0	0.6 \pm 0.1	2.3 \pm 0.2	0.5 \pm 0.0	1.6 \pm 0.2
7	0.9 \pm 0.0	1.4 \pm 0.1	0.7 \pm 0.0	3.0 \pm 0.4	0.7 \pm 0.0	1.8 \pm 0.0	0.6 \pm 0.0	2.3 \pm 0.1	1.1 \pm 0.1	1.8 \pm 0.0	0.8 \pm 0.1	2.0 \pm 0.1	0.8 \pm 0.0	1.6 \pm 0.1
8	1.2 \pm 0.0	1.4 \pm 0.0	1.0 \pm 0.0	2.2 \pm 0.0	1.0 \pm 0.0	1.7 \pm 0.1	0.9 \pm 0.0	1.9 \pm 0.1	1.3 \pm 0.0	1.7 \pm 0.0	1.1 \pm 0.1	1.8 \pm 0.0	1.1 \pm 0.0	1.6 \pm 0.2
16	1.6 \pm 0.0	1.3 \pm 0.0	1.6 \pm 0.0	1.8 \pm 0.1	1.6 \pm 0.0	1.4 \pm 0.1	1.5 \pm 0.0	2.0 \pm 0.2	1.5 \pm 0.0	1.9 \pm 0.1	1.5 \pm 0.0	2.0 \pm 0.3	1.6 \pm 0.0	1.5 \pm 0.2

Table 5. GMF and OD₆₂₀ readings for yeast forms of the PALS-GFP strains grown in YNB medium

Details as for Table 4.

Time (h)	CAI12		PALS1-GFP		PALS3-GFP		PALS5-GFP		PALS6-GFP		PALS7-GFP		PALS9-GFP	
	OD	GMF												
1	0.04 \pm 0.00	2.0 \pm 0.1	0.04 \pm 0.00	52 \pm 2.4	0.04 \pm 0.00	1.8 \pm 0.0	0.04 \pm 0.00	2.4 \pm 0.1	0.04 \pm 0.00	2.1 \pm 0.3	0.04 \pm 0.00	1.8 \pm 0.2	0.04 \pm 0.00	2.4 \pm 0.3
2	0.04 \pm 0.00	1.7 \pm 0.0	0.04 \pm 0.00	39 \pm 1.1	0.04 \pm 0.00	1.9 \pm 0.1	0.04 \pm 0.00	2.2 \pm 0.0	0.05 \pm 0.00	2.2 \pm 0.2	0.04 \pm 0.00	1.9 \pm 0.1	0.04 \pm 0.00	2.6 \pm 0.4
3	0.05 \pm 0.00	2.1 \pm 0.0	0.05 \pm 0.00	37 \pm 1.8	0.05 \pm 0.00	2.3 \pm 0.1	0.05 \pm 0.00	2.1 \pm 0.0	0.06 \pm 0.00	2.3 \pm 0.1	0.05 \pm 0.00	2.1 \pm 0.1	0.05 \pm 0.00	2.5 \pm 0.3
4	0.07 \pm 0.00	2.2 \pm 0.1	0.06 \pm 0.00	26 \pm 2.3	0.06 \pm 0.00	2.7 \pm 0.3	0.06 \pm 0.00	2.1 \pm 0.0	0.07 \pm 0.00	2.3 \pm 0.1	0.05 \pm 0.00	2.6 \pm 0.0	0.06 \pm 0.00	2.7 \pm 0.3
5	0.10 \pm 0.00	1.9 \pm 0.2	0.08 \pm 0.00	16 \pm 2.0	0.09 \pm 0.01	2.2 \pm 0.3	0.09 \pm 0.01	2.2 \pm 0.1	0.10 \pm 0.01	2.2 \pm 0.1	0.08 \pm 0.01	2.5 \pm 0.0	0.10 \pm 0.01	3.1 \pm 0.3
6	0.15 \pm 0.01	1.8 \pm 0.0	0.13 \pm 0.01	9.7 \pm 0.9	0.15 \pm 0.01	2.0 \pm 0.1	0.13 \pm 0.01	2.4 \pm 0.0	0.18 \pm 0.01	2.5 \pm 0.1	0.11 \pm 0.02	2.6 \pm 0.2	0.16 \pm 0.02	2.4 \pm 0.4
7	0.25 \pm 0.02	1.5 \pm 0.0	0.21 \pm 0.01	5.8 \pm 0.2	0.25 \pm 0.02	1.8 \pm 0.1	0.20 \pm 0.02	2.6 \pm 0.2	0.29 \pm 0.02	2.5 \pm 0.2	0.16 \pm 0.03	3.0 \pm 0.0	0.27 \pm 0.03	2.5 \pm 0.4
8	0.45 \pm 0.03	1.6 \pm 0.1	0.35 \pm 0.03	4.4 \pm 0.1	0.43 \pm 0.04	1.6 \pm 0.0	0.33 \pm 0.03	2.7 \pm 0.3	0.49 \pm 0.03	2.5 \pm 0.1	0.26 \pm 0.05	3.4 \pm 0.6	0.45 \pm 0.05	2.4 \pm 0.3
9	0.73 \pm 0.03	1.6 \pm 0.0	0.54 \pm 0.03	4.4 \pm 0.1	0.68 \pm 0.04	1.5 \pm 0.0	0.53 \pm 0.04	2.3 \pm 0.1	0.72 \pm 0.03	2.5 \pm 0.1	0.38 \pm 0.07	3.3 \pm 0.2	0.60 \pm 0.03	2.1 \pm 0.5
10	0.96 \pm 0.03	1.7 \pm 0.1	0.70 \pm 0.03	3.3 \pm 0.5	0.92 \pm 0.03	1.7 \pm 0.0	0.76 \pm 0.04	2.7 \pm 0.2	0.97 \pm 0.02	3.4 \pm 0.4	0.62 \pm 0.10	4.0 \pm 0.2	0.91 \pm 0.07	2.2 \pm 0.5
16	1.23 \pm 0.01	2.7 \pm 0.4	1.11 \pm 0.02	6.3 \pm 0.2	1.22 \pm 0.01	2.3 \pm 0.4	1.10 \pm 0.02	3.2 \pm 0.1	1.03 \pm 0.05	2.8 \pm 0.8	0.95 \pm 0.06	3.9 \pm 0.9	1.08 \pm 0.04	2.7 \pm 0.6

Table 6. GMF readings for the PALS7-GFP reporter strain grown as yeast forms in YPD medium

Fluorescence intensity of budding cells was measured at the indicated time points by flow cytometry. GMF values are reported in arbitrary units. Values are the mean \pm SEM of GMF values from three separate experiments.

Time (min)	GMF	
	CAI12	PALS7-GFP
0	1.4 \pm 0.0	1.9 \pm 0.0
20	1.9 \pm 0.1	3.1 \pm 0.1
40	2.3 \pm 0.1	3.3 \pm 0.1
60	2.3 \pm 0.1	3.5 \pm 0.3
80	2.6 \pm 0.2	3.2 \pm 0.4
100	2.7 \pm 0.1	3.9 \pm 0.5
120	2.7 \pm 0.1	4.2 \pm 0.4
140	2.7 \pm 0.2	5.0 \pm 0.2
160	2.4 \pm 0.1	4.5 \pm 0.1
180	2.2 \pm 0.1	3.7 \pm 0.1
200	1.9 \pm 0.0	2.8 \pm 0.0
220	1.8 \pm 0.1	2.4 \pm 0.0
240	2.0 \pm 0.1	2.3 \pm 0.0

the ALS9 promoter, which were sufficient to produce GFP-specific transcript by RT-PCR (Fig. 1).

A real-time RT-PCR assay was developed to provide additional evidence that the PALS-GFP clones reflected the expression from native ALS promoters. A culture of strain CAI12 was grown in YPD for 16 h at 37 °C, total RNA extracted and real-time RT-PCR performed. Although ALS expression was expected to be low at this time point (Table 4), this growth condition is used commonly to produce inoculum cells for disease models in the laboratory. As such, cells grown in this manner have been evaluated frequently using our standard RT-PCR assay (Green *et al.*, 2004), providing additional data for comparison. Transcript copy numbers were 170 \pm 29 for ALS1, 310 \pm 33 for ALS3, 37 \pm 5.1 for ALS5, 0.1 \pm 0.1 for ALS6, 1.7 \pm 0.8 for ALS7 and 450 \pm 110 for ALS9, compared to 230 000 \pm 55 000 for

the *TEF1* housekeeping gene. Transcript copy numbers were also measured for ALS2 (7300 \pm 180) and ALS4 (220 \pm 76).

The PALS-GFP reporter strains grown in YNB + Glc (Table 5) showed generally similar GMF readings to those from YPD-grown cells. However, some notable exceptions were observed. For example, in YNB, ALS1 transcription did not reach the levels observed in YPD. The PALS7-GFP strain did not show the transient increase in GMF values around 3 h, but instead showed a slight steady increase as the culture aged. The PALS9-GFP strain had GMF readings that were more elevated relative to the control compared to the values observed when the strain was grown in YPD. Overall, the control and reporter strains grew more slowly and to a lower density in YNB than in YPD.

Flow cytometry analysis of *C. albicans* PALS-GFP germ tubes

The PALS-GFP reporter strains were assayed for fluorescence changes during germ tube formation in RPMI at 37 °C (Table 7) and in YPD with 10 % serum (Table 8). Germ tube growth was faster in the serum-containing medium, so instead of measuring fluorescence every 15 min for 1 h as for RPMI-grown cells (Table 7), measurements were taken every 10 min for 0.5 h (Table 8). In both media, PALS1-GFP was the earliest and most strongly up-regulated gene, followed both temporally and in intensity by PALS3-GFP. PALS3-GFP expression increased dramatically when germ tubes became visible by microscopic observation. GMF readings for the PALS5-GFP, PALS6-GFP, PALS7-GFP and PALS9-GFP strains were similar to those for the CAI12 negative control, suggesting at most, low levels of promoter activity under the growth conditions tested. Real-time RT-PCR measurements of transcript copy number supported the flow cytometry results. Transcript copy numbers were 500 000 \pm 36 000 for ALS1, 490 000 \pm 180 000 for ALS3, 110 \pm 32 for ALS5, 5.6 \pm 0.8 for ALS6, 6.0 \pm 2.8 for ALS7 and 490 \pm 120 for ALS9, compared to 3 600 000 \pm 530 000 for the *TEF1* housekeeping gene. Transcript copy numbers were also measured for ALS2 (180 000 \pm 9900) and ALS4 (1500 \pm 590). Kinetics of germ tube formation differed between the RPMI-grown PALS-GFP cells in the flow

Table 7. GMF readings for PALS-GFP germ tubes grown in RPMI 1640

Fluorescence intensity during germ tube formation was analysed every 15 min over a 1 h incubation in RPMI at 37 °C. Values are the mean \pm SEM of GMF values taken from three separate experiments. Percentage germ tubes were counted in each 1 h culture in order to ensure that the PALS-GFP strains were not defective in filamentation when grown in this medium. The percentage of germ tubes at the 1 h time point was between 90 and 99 % for all strains, including the wild-type control.

Time (min)	CAI12	PALS1-GFP	PALS3-GFP	PALS5-GFP	PALS6-GFP	PALS7-GFP	PALS9-GFP
0	1.2 \pm 0.0	1.4 \pm 0.0	1.2 \pm 0.0	1.3 \pm 0.1	1.4 \pm 0.1	1.3 \pm 0.1	1.4 \pm 0.0
15	1.8 \pm 0.0	16 \pm 1.0	1.8 \pm 0.0	1.9 \pm 0.1	2.0 \pm 0.1	2.0 \pm 0.0	1.9 \pm 0.1
30	1.9 \pm 0.1	48 \pm 1.2	3.6 \pm 0.6	2.0 \pm 0.2	1.9 \pm 0.0	2.1 \pm 0.2	1.9 \pm 0.0
45	2.0 \pm 0.1	69 \pm 1.2	13 \pm 3.1	2.1 \pm 0.3	1.8 \pm 0.0	2.1 \pm 0.3	1.9 \pm 0.0
60	2.1 \pm 0.1	78 \pm 1.0	26 \pm 6.0	2.3 \pm 0.2	1.9 \pm 0.1	2.3 \pm 0.1	2.1 \pm 0.0

Table 8. GMF readings for PALS-GFP germ tubes grown in YPD with 10% serum

Fluorescence intensity during germ tube formation was analysed every 10 min over a 30 min incubation in YPD with 10% serum at 37 °C. Other details as for Table 7.

Time (min)	CAI12	PALS1-GFP	PALS3-GFP	PALS5-GFP	PALS6-GFP	PALS7-GFP	PALS9-GFP
0	1.3±0.0	1.8±0.1	1.4±0.0	1.7±0.3	1.4±0.0	1.3±0.0	1.3±0.0
10	1.6±0.1	6.4±0.6	1.8±0.0	1.8±0.0	2.0±0.3	1.9±0.0	1.7±0.2
20	2.2±0.4	39±1.6	2.9±0.1	2.5±0.1	2.3±0.0	3.3±0.5	2.3±0.2
30	2.9±0.2	86±1.4	11±0.7	3.7±0.3	3.3±0.1	3.6±0.2	3.7±0.5

cytometry experiment and those of strain CAI12 used for real-time RT-PCR analysis. Microscopic evaluation of germ tube length for both culture conditions showed that germ tubes on the cells from the real-time analysis were longer than those from the flow cytometry analysis that had been grown for the same amount of time. The PALS-GFP reporter strains were matched to CAI12 for growth rate and did not show differences in germ tube formation when incubated in the same experiment. The only difference between the experiments was the size of the culture flask used (50 ml for reporter strains in flow cytometry vs 500 ml for CAI12 for real-time analysis), and this may have contributed to the quicker germ tube formation for the wild-type strain. These growth rate differences may have also accounted for the different patterns of relative abundance of *ALS1* and *ALS3* transcripts from the flow cytometry data (where the values appeared different) and the real-time analysis (where the values were the same). Regardless of these differences between the flow cytometry and real-time RT-PCR analyses, the main conclusions from the two methods supported each other and indicated that some ALS genes were transcribed at high levels while others were relatively quiet.

DISCUSSION

The construction of PALS-GFP reporter strains and the evaluation of their fluorescence by flow cytometry was pursued because we sought an inexpensive method that would provide a dynamic, quantitative assessment of ALS gene expression in living cells. To demonstrate that ALS gene expression in the reporter strains was the same as in wild-type *C. albicans*, we also developed a real-time RT-PCR assay. Results from testing the PALS-GFP reporter strains at specific time points were similar to those for strain CAI12 and validated the use of the reporter strains for further analysis. While quantitative assessments of ALS gene expression can be made using the real-time RT-PCR method, this technology is far more expensive and time-consuming than flow cytometry analysis.

The PALS-GFP reporter strains were used to follow ALS gene expression over time in two cultures where yeast forms were grown and two cultures that yielded germ tubes. The overall conclusion from these analyses was that the ALS family has genes that are regulated by large increases and decreases in transcriptional activity and also those that

show a more consistent low-level activity. Although *ALS1* expression has been discussed in previous publications (Hoyer *et al.*, 1995; Fu *et al.*, 2002; Zhao *et al.*, 2004), this report is believed to be the first to follow *ALS1* expression over a growth curve. Results for all media tested showed that *ALS1* expression increases dramatically when cells are placed into fresh medium, whether the conditions promote growth as yeast or hyphal forms. These observations suggest that Als1p functions in response to new environments or perhaps in the release of cells from stationary phase.

Studies here also corroborated the hypha-specific regulation of *ALS3*. This relationship between the morphological form and *ALS3* transcription was demonstrated originally by Northern blotting (Hoyer *et al.*, 1998a). *ALS3* transcription increases when germ tubes are visible microscopically, in contrast to transcription of *ALS1*, which occurs almost immediately after *C. albicans* cells are placed into the hyphal-induction media tested (Zhao *et al.*, 2004). This pattern of regulation suggests that Als1p is localized at the initial point where the germ tube emerges from the mother yeast, a localization that has been demonstrated by immunostaining methods (Fu *et al.*, 2002). Localization of Als3p awaits production of a specific antiserum or utilization of an alternative method, but from the data here, it is likely that Als3p will be localized farther down the germ tube length than Als1p. Direct comparisons between *als1Δ/als1Δ* and *als3Δ/als3Δ* strains in adhesion to vascular endothelial and buccal epithelial cells showed the dramatic contribution to *C. albicans* adhesion by Als3p (Zhao *et al.*, 2004). Hypha-specific production of Als3p helps to explain the increased adhesive capacity of germ tubes relative to yeast forms (Calderone & Braun, 1991). The GFP strains described here could be used to study ALS gene expression during prolonged filamentation, although an alternative technique such as fluorescence microscopy would have to be used since longer filamentous forms are not amenable to flow cytometry analysis.

Construction of the PALS2-GFP strain was not completed, consistent with previous observations that integration of constructs at the *ALS2* locus is problematic (Zhao *et al.*, 2005). However, the real-time RT-PCR data showed that *ALS2* transcription is stronger than transcription of the other ALS genes at 16 h in YPD-grown cells and that *ALS2*

transcription is strongly up-regulated during germ tube formation in both RPMI and serum-containing media. The YPD result from this analysis matches previous observations that the *ALS2* message is one of the last to disappear from RT-PCR analysis of a dilution series of cDNA made from an overnight culture of YPD-grown *C. albicans* cells (Green *et al.*, 2004). In that previous work, the *ALS1* transcript appeared equally as strong as that from *ALS2*. The disparity between *ALS1* results from the two studies can be explained by noting that *ALS1* transcription increases when YPD-grown cells are washed in PBS (C. B. Green & L. L. Hoyer, unpublished observation). The PBS washing step was included in the previous analysis (Green *et al.*, 2004), but not in the analysis described in this paper. Transcript copy number also increased for *ALS4* during germ tube formation in RPMI, although not to the same magnitude as for *ALS2* transcription.

In comparison with the strong transcriptional responses described above, those from other genes were relatively mild, with *ALS6* and *ALS7* showing the lowest transcriptional activity within the family. This result matches previous studies where *ALS6* and *ALS7* expression most frequently fell below the detection limit for our standard RT-PCR assay (Green *et al.*, 2004). The low level of transcription for *ALS6* and *ALS7*, and also for *ALS5*, limits the value of the PALS-GFP reporter strains and flow cytometry for assessment of transcriptional activity. In some cases, the transcript copy numbers are so low that it is questionable whether these genes are really active at all under the conditions tested. Work by Zhang *et al.* (2003) showed *ALS7* message on a Northern blot of 20 µg poly(A) RNA, consistent with the conclusion of low-level transcriptional activity in cultures similar to our YPD growth conditions. In data presented here, GFP transcript could be amplified by RT-PCR from all of the PALS-GFP reporter strains, consistent with the conclusion of activity from each of the ALS promoters. Despite the low level of transcript, the signal from the *ALS7* promoter increased in a statistically significant manner following 2 h growth in fresh YPD medium. These growth conditions can be exploited to learn more about Als7p function by microarray analysis of *als7/als7* mutant strains. Expression data for the other ALS genes will be used similarly.

Despite the technical differences between the various approaches to studying ALS gene expression, the results from the methods are very similar. If the level of transcription is positively associated with protein production, the data would suggest that much less Als5p, Als6p and Als7p will be present on the *C. albicans* cell surface than Als1p, Als2p or Als3p. Reagents and methods that can discriminate between the various Als proteins are required to test this hypothesis. Insight into the levels of the various Als proteins that are likely to be present on the *C. albicans* cell surface also provides guidance for the selection of methods to study Als protein function. For example, using overexpression methods to study an Als protein that is

present in small quantities on the *C. albicans* cell might lead to experimental artefacts.

Comparison between data for the various ALS genes also highlights the tight control of ALS expression in *C. albicans*. For example, in the SC5314 background, three ALS genes occupy an approximately 20 kb contiguous region of chromosome 6 where *ALS1* is localized between *ALS5* and *ALS9* (Zhao *et al.*, 2003). Considering the transcriptional abundance from the *ALS1* locus, minimal expression of *ALS5* and *ALS9* illustrate the fine regulation of ALS gene expression. Functional comparisons can also be inferred from gene expression data. One example is to consider *ALS1*, *ALS3* and *ALS5*, which are more than 85% identical within the 5' domain of each coding region. By comparison with data from studies of *Saccharomyces cerevisiae* α -agglutinin (Wojciechowicz *et al.*, 1993), this region of the ALS gene has been proposed to encode the Als adhesive functional domain (Hoyer, 2001). Gene expression data suggest that high levels of Als1p are present on newly budding yeast forms and newly forming germ tubes, high levels of Als3p are deposited on the surface of elongating germ tubes, and scant amounts of Als5p potentially are present on either growth form. These data suggest that there is a need for large amounts of Als1p as cells begin to divide, large amounts of Als3p as germ tubes lengthen, and only small amounts of Als5p throughout growth under the conditions tested. These functional clues will be combined with information from other approaches to further define the roles of each Als protein and functional interrelationships within the Als family.

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