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Ssn6, an important factor of morphological conversion and virulence in *Candida albicans*

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Summary

***Candida albicans*, the major fungal pathogen in humans, undergoes morphological conversion from yeasts to filamentous growth forms depending upon various environmental conditions. Here, we have identified a *C. albicans* gene, namely *SSN6*, encoding a putative global transcriptional co-repressor that is highly homologous to the *Saccharomyces cerevisiae* Ssn6. The isolated *C. albicans SSN6* complemented the pleiotropic phenotypes of *S. cerevisiae ssn6* mutation, and its expression levels declined significantly in response to a strong true hyphal inducer, serum. The mutant lacking *C. albicans* Ssn6 displayed a stubby pseudohyphal growth pattern, derepressed filament-specific genes in response to elevated temperature 37°C and failed to develop true hyphae, extensive filamentation and virulence. Such morphological defects of *ssn6*/*ssn6* mutant were not rescued by overexpression of Tup1, Cph1 or Efg1. Moreover, epistatic analysis showed that, as far as cell morphology was concerned, Ssn6 was epistatic to Tup1 at the higher temperature but that, at the lower temperature, the *ssn6*/*ssn6 tup1*/*tup1* double mutant grew in a stubby form of pseudohyphae distinct from the phenotypes of either single mutant. Furthermore, overexpression of *SSN6* in *C. albicans* led to enhanced filamentous growth and attenuated virulence. These findings suggest that Ssn6 may function as an activator as well as a repressor of filamentous growth and be a target for candidacidal drugs, as its excess or deficiency resulted in impaired virulence.**

Introduction

Candida albicans is the most frequently encountered fungal pathogen in humans. It causes not only the oral and vaginal thrush, but also systemic or life-threatening infections in immunocompromised hosts such as leukaemic, diabetic, organ transplant and human immunodeficiency virus-infected patients (Odds *et al*., 1988; Cutler, 1991; Corner and Magee, 1997). One of the virulence properties of *C. albicans* has been attributed to its ability to undergo reversible morphological conversion between yeasts, true hyphae (elongated thread-like tubes with parallel sides and no constriction at the septa) and pseudohyphae (a variety of shapes from attached strings of yeast-like cells to long filaments with constriction at the septa) (Odds, 1985; Brown and Gow, 1999). These flexible morphological spectra are thought to be required for adaptation in warm-blooded hosts, rapid colonization of tissues and facilitated spread of infection.

The morphological conversion from yeast to filamentous growth forms (true hyphae or pseudohyphae) in *C. albicans* is regulated by several conserved signalling pathways such as a Cph1-mediated mitogen-activated protein kinase (Liu *et al*., 1994) and an Efg1-mediated cyclic AMP/protein kinase A (cAMP/PKA) (Lo *et al*., 1997; Stoldt *et al*., 1997; Brown and Gow, 1999; Ernst, 2000). In addition, a transcription repressor Tup1 controls the filamentous growth of *C. albicans*, as the *tup1/tup1* mutant grows exclusively as filaments (predominantly pseudohyphae) (Braun and Johnson, 1997).

In *Saccharomyces cerevisiae*, Tup1 forms a transcription co-repressor complex in concert with Ssn6 and regulates a diverse set of genes controlled by mating type, glucose, oxygen and DNA damage (Keleher *et al*., 1992; Treitel and Carlson, 1995; Smith and Johnson, 2000). Either *tup1* or *ssn6* mutant shows pleiotropic phenotypes such as loss of cell type control in cells, flocculation, loss of glucose repression, defective growth at 37°C or in glycerol, etc. (Smith and Johnson, 2000). Although neither Tup1 nor Ssn6 itself has any DNA-binding activity, they are recruited to a specific promoter through the interaction with distinct upstream DNA-binding proteins such as Mig1, Rox1, Ctr1 and Nrg1. Once the Ssn6–Tup1 complex has been attracted to a promoter, it is presumed to impose transcriptional repression through a direct interaction with transcriptional machinery, histone proteins and transcriptional activators (Smith and Johnson, 2000).

Ssn6 is a representative protein containing tetratri-

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copeptide repeat (TPR) motifs that are known to mediate protein–protein interaction (Goebl and Yanagida, 1991). In the Ssn6–Tup1 complex, Ssn6 functions as an adapter to stabilize interactions between Tup1 and various target proteins, whereas Tup1 bears the transcriptional repression activity of the complex via direct interaction with histone proteins or basal transcriptional machinery (Keleher *et al*., 1992; Tzamarias and Struhl, 1994; Edmondson *et al*., 1996).

Recently, the specific DNA-binding proteins including Rfg1 (a close relative of *S. cerevisiae* Rox1; Kadosh and Johnson, 2001; Khalaf and Zitomer, 2001) and Nrg1 (Braun *et al*., 2001; Murad *et al*., 2001a) have identified as repressors for filamentous growth in *C. albicans*. Therefore, Tup1 is postulated to form a complex with Ssn6 and to repress filamentous growth in *C. albicans* similar to the Ssn6–Tup1 complex of *S. cerevisiae* (Braun and Johnson, 1997; Braun *et al*., 2001; Kadosh and Johnson, 2001; Murad *et al*., 2001a,b). However, the obvious roles of Ssn6, its partner protein, are not yet known in *C. albicans*. In the present study, we provide the interesting results that Ssn6 may function not only as a repressor but also as an activator for filamentous growth and be required for virulence in *C. albicans*.

Results

Isolation and characterization of SSN6

To investigate the roles of Ssn6 in *C. albicans*, we isolated the gene from a *C. albicans* ATCC 10231 genomic library (Hwang *et al*., 1999) using a sequence trace of *SSN6* from the Stanford *C. albicans* sequencing project (http://www-sequence.stanford.edu/group/candida). The conceptual open reading frame (ORF) of *SSN6* encoded a polypeptide of 1085 amino acids with a calculated molecular mass of 120 540 Da and contained six CUG codons, which encode serine in *C. albicans* but leucine in *S. cerevisiae* and elsewhere (Santos *et al*., 1993). The predicted amino acid sequence of *C. albicans* Ssn6 showed 38% identity with *S. cerevisiae* Ssn6 over the entire amino acid sequence (Schultz and Carlson, 1987). Like *S. cerevisiae* Ssn6, the *C. albicans* Ssn6 contains 10 copies of the consecutive TPR motif (70% identity with those of *S. cerevisiae* Ssn6), glutamine- or proline-rich regions and putative cAMP-dependent phosphorylation sites at similar positions (Fig. 1). Generally, the proteins containing TPR motifs are known to be involved in transcriptional repression, cell cycle control, stress responses and protein transport (Goebl and Yanagida, 1991). In *S. cerevisiae* Ssn6, TPR motifs mediate the interaction with Tup1 and specific DNA-binding proteins for transcription repression (Tzamarias and Struhl, 1994; Smith and Johnson, 2000). The glutamine- and proline-rich regions are frequently observed in transcription factors and are thought to mediate transcription activation through protein–protein interactions (Courey and Tjian, 1988; Mermod *et al*., 1989). Moreover, cAMP-dependent phosphorylation sites are presumably phosphorylated via PKA, the levels of which are important for the control of growth, stress response, morphological conversion and virulence in pathogenic fungi (Bockmühl *et al*., 2001; D'Souza and Heitman, 2001). When compared with the deduced amino acid sequence of an *SSN6* candidate recently uploaded from the *Candida* genome project, that of the cloned *SSN6* was found to have 99% identity to the contig-6 ORF 6.8833 (http://www-sequence.stanford.edu:8080/contigblast/contigs6/8/seqs/orf6.8833.prot). Thus, to confirm whether or not there is another gene coding *SSN6* in *C. albicans*, Southern analysis was performed with genomic DNAs from CA10231 and SC5314 strains. When these genomic DNAs were digested with *Bgl*II, *Nco*I, *Nde*I or *Xba*I, whose restriction sites were not in the *SSN6* coding region, only one band was detected (data not shown). The resulting hybridization pattern indicates that there is a single copy of *SSN6* per *C. albicans* genome. Hence, the observed discrepancies in both sequences may result from strain differences between CA10231 and SC5314 or the fact that heterozygotic alleles might be sequenced, as observed occasionally in the *C. albicans* genome (Saporito-Irwin *et al*., 1995; White, 1997; De Backer *et al*., 2000).

To see whether the *C. albicans* Ssn6 was able to substitute functionally for *S. cerevisiae* Ssn6, it was expressed in an *S. cerevisiae ssn6* mutant. The mutant cells carrying *C. albicans SSN6* partially restored repression of glucose, grew rapidly and rescued defective growth at 37°C or in glycerol, indicating that several phenotypic characteristics in the *S. cerevisiae ssn6* mutant had been complemented by the *C. albicans SSN6* (Table 1). Furthermore, the overexpressed *C. albicans SSN6* restored repression of a genomic α-specific gene reporter, *STE6-PHO5*, that is not expressed in α cells, but normally in **a** cells (Mukai *et al*., 1999).

Downregulation of SSN6 *transcript during true hyphal development*

To examine *SSN6* expression in response to serum, a strong true hyphal inducer, exponentially growing wild-type SC5314 cells were cultured in 10% serum + YPD medium at 37°C. Surprisingly, Northern analysis revealed that the levels of *SSN6* expression declined significantly during serum-induced true hyphal development (Fig. 2). After 30 min in this medium, 45% of cells initiated to form germ tubes, but the level of *SSN6* mRNA was unchanged. However, after 1 h, all cells produced germ tubes, and the level of *SSN6* transcript declined drastically. Then, during

A

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CaSsn6 : MYATAHTIKQQQQQHPPPPPLNGGLHASGAPPNSHEAAAIAQQQQQQQQHHNGPGMIVAAAAASANQQAVQARAQQQQQQQQQQ :   84
ScSsn6 : -------------MNP------GGEQTIMEQP--------AQQQQQQQ------------------------QQQQQQQQQA :   31

CaSsn6 : RLPSS--AALNETTVSTWLAIGSLAESLGDIERATASYNSALRHSPNNPDILVKIANTYRSKDQFLKAAELYEQALNFHVENGE :  166
ScSsn6 : AVPQQPLDPLTQSTAETWLSIASLAETLGDGDRAAMAYDATLQFNPSSAKALTSLAHLYRSRDMFQRAAELYERALLVNPELSD :  115

CaSsn6 : TWGLLGHCYLMLDDLQRAYAAYQRALFYLENPNVPKLWHGIGILYDRYGSLEYAEEAFVRVLDLDPNFDKANEIYFRLGIIYKH :  250
ScSsn6 : VWATLGHCYLMLDDLQRAYNAYQQALYHLSNPNVPKLWHGIGILYDRYGSLDYAEEAFAKVLELDPHFEKANEIYFRLGIIYKH :  199

CaSsn6 : QGKLQPALECFQYILNNPPHPLTQPDVWFQIGSVYEQQKDWNGAKDAYEKVLQINPHHAKVLQQLGCLYSQAESNPPTPANGAA :  334
ScSsn6 : QGKWSQALECFRYILPQPPAPLQEWDIWFQLGSVLESMGEWQGAKEAYEHVLAQNQHHAKVLQQLGCLYGMSNVQFYDPQK---  :  280

CaSsn6 : QPHKPFQQDLTIALKYLKQSLEVDQSDAHSWYYLGRVEMIRGDFTAAYEAFQQAVNRDARNPTFWCSIGVLYYQISQYRDALDA :  418
ScSsn6 : ------------ALDYLLKSLEADPSDATTWYHLGRVHMIRTDYTAAYDAFQQAVNRDSRNPIFWCSIGVLYYQISQYRDALDA :  352

CaSsn6 : YTRAIRLNPYISEVWYDLGTLYETCNNQISDALDALDAYRQAERLDPNNPHIKARLEQLTKYQQ-EGNTHPPQPPPSSQQPRLP :  501
ScSsn6 : YTRAIRLNPYISEVWYDLGTLYETCNNQLS---DALDAYKQAARLDVNNVHIRERLEALTKQLENPGNINKSNGAPTNASP-AP :  432

CaSsn6 : QGMVLESTQQQQQQQQQQPPPPPPPQQLQHQSQSQSQPQPQQPPQIQSQPSLLQHQSSLPPQQIQPLHQQAAKPLVNQQQSPP :  585
ScSsn6 : PPVILQPTLQPNDQG--NPLNTRISAQSANATASMVQQQHPAQQTPINSS-ATMYSNGASPQLQAQAQAQAQAQAQAQAQAQ :  513

CaSsn6 : PPHLMNLGQPGQQPRQLPPHLPPHTQQPSQIQEKPPTQEQPHHQPPPPPQHQQQSQSQPQPPHQPQHTQNQSPQLAQLPPHHSN :  669
ScSsn6 : AQAQAQAQAQAQAQAQAQAQAQAHAQAQAQAQAKAQAQAQAQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQLQPLPRQQ-- :  595

CaSsn6 : PPANPHGAPQQRTGLPDLLHNSANIISAPSQVPQPQQQYQQPHIAPVRQEQVNHVPSIYSAPRPTETTLPQINNPNESTTTQVP :  753
ScSsn6 : ---------LQQKGVSVQMLN-------PQQG-QP--YITQPTVIQAHQLQP---FSTQAMEHPQSSQLPPQQQ--QLQSVQHP :  655

CaSsn6 : QLKKEEPKPEATVSAPVPEAIKVQDQVTIQESAPAAAAVSAPASAPVGDIKTDTVSTTTPATSTTADAVPVSVSQVGEAPNVVQ :  837
ScSsn6 : QQLQGQPQ----AQAPQPLIQHNVEQNVLPQKRYMEGAIHTLVDAAVSSSTHTENNTKSPRQPTHAIPTQAPATGITNAEPQVK :  735

CaSsn6 : EKKVPDTEQIVSQVEKPVESQPE--VTPAPTPAPALATAPTEPAPTDKDVVMAPSKSATPIPQSIVEQNTRVSEATKAPESNGK :  919
ScSsn6 : KQKLNSPNSNINKLVNTATSIEENAKSEVSNQSPAVVESNTNNTSQEEKPVKANSIPSVIGAQEPPQEASPAEEATKAASVSPS :  819

CaSsn6 : HDLEDKNDEEKILKRPTVETTTESVPVNQPVEKENEKVEVPPPPEQPSSEKREKEVNGSIKKPLENESKVDIPQFSSNITAQNE : 1003
ScSsn6 : TKPLNTE-PESSSVQPTVSSESSTTKANDQSTAETIELSTATVPAEASP--VEDEVRQHSKE--EN----GTTEASAPSTEEAE :  894

CaSsn6 : EAKSGEETKKDTTKTSPAKQGEVKEVIPSSTETVSKPDVEKDNKEKDKDEDEVMADEDDVKKDENPEPPMRKIEEDENYDDE    : 1085
ScSsn6 : PAAS-----RDAEKQQDETAATTITVIKPTLETMETVKEEAKMREEEQTSQEKSPQENTLPR-EN---VVRQVEEDENYDD-    :  966
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B

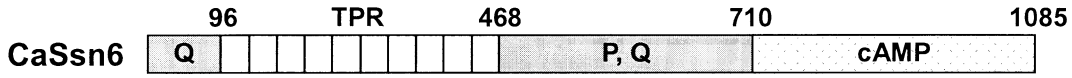

Fig. 1. Sequence of *C. albicans* Ssn6.
A. Alignment of *C. albicans* Ssn6 (CaSsn6) and *S. cerevisiae* Ssn6 (ScSsn6) was performed by the programs CLUSTALW and GENDOC. Identical residues are shadowed, TPR regions boxed and cAMP-dependent phosphorylation sites underlined.
B. Schematic representation of the primary structures of the Ssn6. Ten tandem copies of TPR, glutamine-rich (Q), proline- and glutamine-rich (P, Q) and putative cAMP-dependent phosphorylation (cAMP) regions are represented.

the transition from germ tubes into true hyphae, *SSN6* mRNA was hardly detected. Considering that the expression of *NRG1* mRNA is reduced during true hyphal growth (Braun *et al*., 2001; Murad *et al*., 2001a), we would predict that downregulation of mRNAs of the genes encoding Ssn6 as well as DNA-binding proteins might be associated with the process of true hyphal development in *C. albicans*. On the other hand, Western analysis using α-Tup1 revealed that Tup1 was increased after 1 h in 10% serum + YPD medium at 37°C (data not shown), indicating that true hyphal development progressed through more complicated mechanisms than simple decline in the repressor complex components.

The mutant lacking C. albicans *Ssn6 was trapped in a stubby pseudohyphal growth pattern and failed to develop true hyphae*

To clarify the roles of Ssn6 in *C. albicans*, both copies of *SSN6* were sequentially disrupted using homologous recombination (see Experimental procedures). *C. albicans* cells lacking Ssn6 showed similar pleiotropic effects to the *S. cerevisiae ssn6* mutant (Márquez *et al*., 1998; Smith and Johnson, 2000): slow growth in non-fermentable carbon sources (acetate or lactate), thermosensitivity at 43°C and enhanced resistance to oxidants such as hydrogen peroxide and menadione (data

Table 1. Complementation of *C. albicans SSN6* in *S. cerevisiae ssn6* mutation.

Strains	Wild type	*ssn6* (vector)	*ssn6* (*CaSSN6*)
Growth rate (doubling time = min)[a]	44.9 ± 1.5	165.9 ± 6.3	55.4 ± 2.1
Invertase activity (U/A_{600})[a]	7.8 ± 2.2	108.5 ± 5.3	26.7 ± 1.7
Growth at 37°C[a]	+	–	+
Growth in glycerol[a]	+	–	+
Acid phosphatase activity (mU/A_{600})[b]	ND[c]	0.77 ± 0.13	0.13 ± 0.01

a. Parental MCY829 and *ssn6* mutant MCY1974 strains were transformed with YEp352 and YEp352-SSN6, and each activity was assayed as described in *Experimental procedures*.
b. *S. cerevisiae ssn6* mutant SH4038-SP harbouring plasmid *STE6-PHO5* was transformed with the plasmids pRS424 and pRS424-SSN6, and then acid phosphatase activities were determined in cell suspensions by standard methods. Values are the average of triplicate determinations ± standard deviation.
c. ND, not determined.

not shown). These observations indicate that Ssn6 of *C. albicans* also seems to share common roles with that of *S. cerevisiae* in response to several stresses.

Interestingly, deletion of *SSN6* also resulted in cells that were essentially trapped in a stubby pseudohyphal growth pattern and were defective in true hyphal development. The *ssn6/ssn6* (CH403) mutant changed colony morphology from normal smooth type to a rough wrinkled one on solid YPD (1% yeast extract, 2% peptone, 2% glucose) medium at 37°C for 3 days, whereas wild-type (SC5314) and heterozygous (CH401) cells maintained a uniformly smooth colony morphology (Fig. 3A). When incubated further for 8 days under the same conditions, wild-type and heterozygote strains developed radial filaments emerging from the edge of colonies, but the *ssn6/ssn6* mutant formed severely wrinkled colonies without any emerging filaments (Fig. 3A). The revertant (CH404R) strain reintroduced with one copy of *SSN6* (see *Experimental procedures*) recovered the ability to develop emerging filaments consistent with the heterozygote strain (Fig. 3A). Microscopic observation of the cells scraped from each colony revealed that the *ssn6/ssn6* mutant had only stubby pseudohyphae with the constriction at the septa, but that wild-type, heterozygote and revertant cells were composed of yeast-form cells and extremely elongated filaments (data not shown).

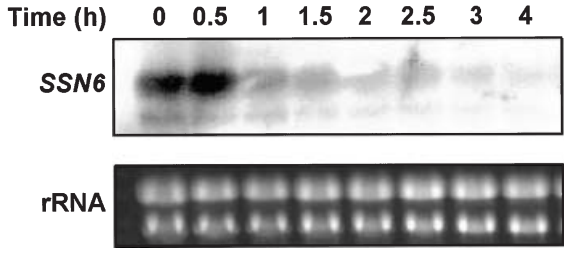

Fig. 2. Downregulation of *SSN6* mRNA during serum-induced true hyphal development. For Northern analysis, RNA was extracted from the wild-type strain (SC5314) after the indicated times of growth in liquid 10% serum + YPD medium at 37°C.

To investigate whether *ssn6* mutation also led to the promoted pseudohyphal growth under true hyphal-inducing conditions, isogenic Ura^+ prototrophs were grown in liquid 10% serum + YPD medium at 37°C. Even under these conditions, the *ssn6/ssn6* mutants grew predominantly as stubby pseudohyphae, whereas wild-type, heterozygote and revertant strains developed elongated true hyphae (Fig. 3B). More interestingly, these pseudohyphae produced by *ssn6* mutation at the higher temperature (37°C) could grow reversibly as yeast-like cells when recultured in YPD medium below 28°C (data not shown). Moreover, the *ssn6/ssn6* mutants formed only stubby pseudohyphae and failed to develop true hyphae or extensive filamentation in various other liquid media or on solid media such as RPMI 1640 (Gibco BRL), corn meal agar (Difco), Spider (Liu *et al*., 1994) or Lee's (Lee *et al*., 1975) in response to elevated temperature (37°C; data not shown).

Effect of ssn6 *mutation on filament-specific gene expression*

To understand the effects of *ssn6* mutation more clearly, we compared the expression of filament-specific genes such as *HWP1* (Staab and Sundstrom, 1998) and *ECE1* (Birse *et al*., 1993) by Northern analysis. As expected, the filament-specific mRNAs were not expressed in the wild-type cells grown in YPD media at 28°C or 37°C. However, these mRNAs were significantly derepressed in the *ssn6/ssn6* cells grown in the same YPD media at the higher temperature of 37°C (Fig. 4), revealing that these expression patterns are consistent with the morphological phenotypes: the promoted pseudohyphal growth at that temperature. It is therefore suggested that Ssn6 is required for the repression of the expression of filament-specific genes at the higher temperature. Under filament-inducing media including 10% serum + YPD, Spider and Lee's, the expression of the filament-specific genes was derepressed in both the wild-type and *ssn6/ssn6* cells, but the expression patterns were different to some extent depending on growth media or genes.

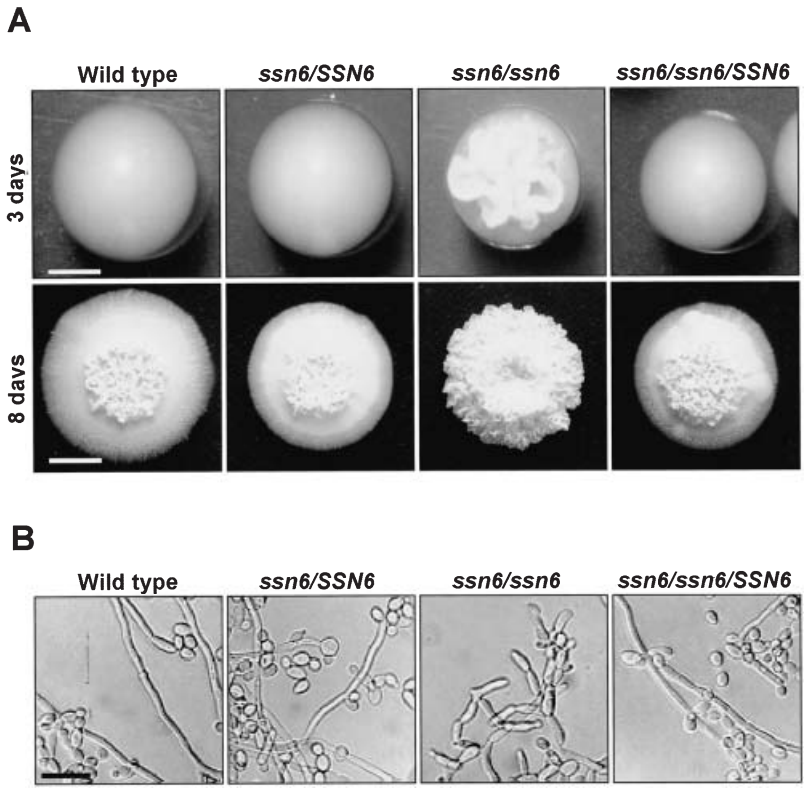

Fig. 3. Colony and cell morphologies of the *ssn6/ssn6* mutant.
A. SC5314 (*SSN6/SSN6*), CH401 (*ssn6/SSN6*), CH403 (*ssn6/ssn6*) and CH404R (*ssn6/ssn6/SSN6*) cells were incubated on YPD solid medium at 37°C for 3 days (scale bar = 1 mm) or 8 days (scale bar = 5 mm) respectively.
B. Promoted pseudohyphal growth and defective hyphal development of *ssn6/ssn6* mutant. Cells were grown in liquid 10% serum + YPD medium at 37°C for 18 h (scale bar = 10 μm).

ssn6 *phenotypes were not suppressed by Tup1, Cph1 or Efg1*

As the *ssn6/ssn6* mutant promoted stubby pseudohyphal growth, we next examined whether overexpression of *TUP1* could suppress this *ssn6* phenotype. When a *TUP1*-overexpressing plasmid (pATUP1) was transformed into wild-type (CAI4) or *ssn6/ssn6* (CH404) strains, the levels of expressed Tup1 in each strain were increased to about 2.3-fold compared with control cells on Western analysis using α-Tup1 (see *Experimental procedures*). However, this overexpression of *TUP1* did not affect the cell morphologies of wild-type and *ssn6/ssn6* strains (Fig. 5A), suggesting that surplus Tup1 alone could not repress the filamentous growth of *C. albicans*.

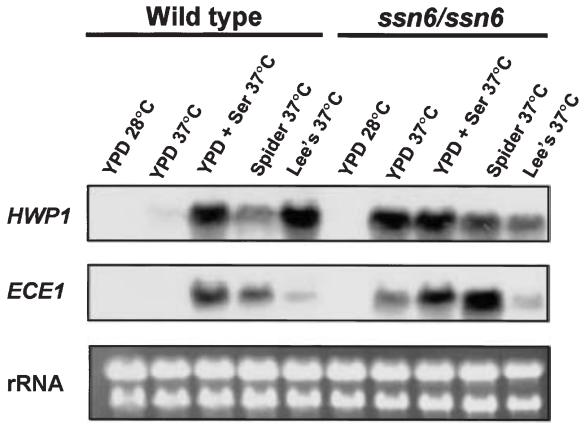

Fig. 4. Expression of filament-specific genes under different conditions in wild-type and *ssn6/ssn6* strains. Cells were diluted from overnight cultures in YPD medium into the media indicated and grown for 3 h in the conditions indicated. Ser, 10% serum. RNAs were prepared from each strain, and Northern analysis was carried out with probes to the indicated genes.

The morphological conversion from yeast to filamentous growth form in *C. albicans* is promoted by at least two regulatory proteins, Cph1 and Efg1. Overexpression of either *CPH1* or *EFG1* shows enhanced filamentous growth (Stoldt *et al*., 1997; Csank *et al*., 1998). We therefore investigated whether Cph1 and Efg1 could rescue the *ssn6* phenotypes in colony and cell morphologies: defects in the emergence of filaments around colonies and in the elongation of stubby pseudohyphae. The resulting overexpression of *CPH1* or *EFG1* could not rescue these *ssn6* phenotypes, whereas that of *CPH1* or *EFG1* in wild type led to extensive filamentation around colonies or extremely elongated filaments respectively (Fig. 5B). Although they failed to rescue *ssn6* defects, the overexpression of *CPH1* or *EFG1* promoted stubby pseudohyphal growth of the *ssn6/ssn6* mutant even at the lower temperature 28°C, where it favoured growth as yeast-like cells (Fig. 5C). This result indicates that both Cph1

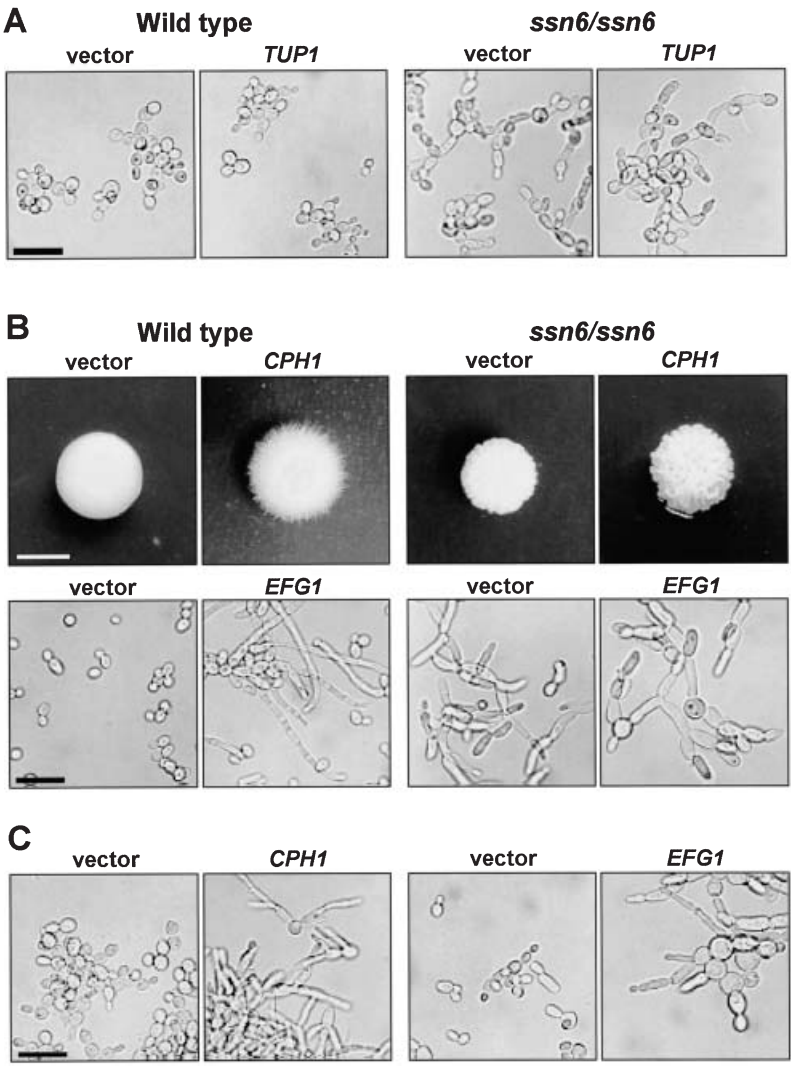

Fig. 5. Overexpression of *TUP1*, *CPH1* or *EFG1* in wild-type and *ssn6/ssn6* strains. Wild-type (CAI4) and *ssn6/ssn6* (CH404) strains were transformed with the control (pYPB1-ADHpL, pBI-1), *TUP1* (pATUP1)-, *CPH1* (pLJ19)- or *EFG1* (pRC2312P-H)-overexpressing vectors. Indicated cells were grown in liquid SD medium at 37°C for 18 h (scale bar = 10 mm) (A), on solid SD medium at 37°C for 7 days (scale bar = 10 mm) or in liquid SCAA medium at 37°C for 18 h (scale bar = 10 μm) (B).
C. The *ssn6/ssn6* (CH404) mutants carrying the empty (pYPB1-ADHpL) or *CPH1*-overexpressing (pLJ19) vectors were incubated on solid SD medium at 28°C for 7 days, scraped from colonies and then observed microscopically. The *ssn6/ssn6* (CH404) mutants carrying the empty (pBI-1) or *EFG1*-overexpressing (pRC2312P-H) vectors were incubated in liquid SCAA medium at 28°C for 18 h (scale bar = 10 μm).

and Efg1 may activate pseudohyphal growth by *ssn6* mutation.

Morphological differences among ssn6/ssn6*,* tup1/tup1 *and* ssn6/ssn6 tup1/tup1 *mutants*

Despite of the common phenotype, such as the promotion of pseudohyphal growth, there are some morphological differences between *ssn6/ssn6* and *tup1/tup1* mutants. In liquid YPD medium, *ssn6* mutation promoted the formation of only stubby pseudohyphae (attached by several thick rod-like cells) in response to elevated temperature, whereas the *tup1/tup1* mutant could grew constitutively as elongated pseudohyphae (attached with long tube-like cells) (Fig. 6A), as reported previously (Braun and Johnson, 1997). Moreover, despite a similar colony morphology (highly wrinkled colony morphology), the *ssn6/ssn6* mutant did not form any filaments emerging from each colony, whereas the *tup1/tup1* mutant displayed many tiny invasive filaments when incubated on solid Spider medium at 37°C for 7 days (Fig. 6B). As a consequence, the *ssn6/ssn6* mutant was less invasive into agar than the *tup1/tup1* mutant. Closer microscopic examination of agar-invasive filaments revealed that those of the *ssn6/ssn6* mutant were composed of stubby pseudohyphae in contrast to extremely elongated pseudohyphae of *tup1/tup1* cells, the same as in liquid medium (Fig. 6B).

Given the different mechanisms by which *S. cerevisiae* Ssn6 interacts with Tup1 and DNA-targeting proteins (Tzamarias and Struhl, 1994; Smith and Johnson, 2000), the phenotypic differences between *ssn6/ssn6* and *tup1/tup1* mutants led us to confirm the possibility that C-terminally truncated *C. albicans* Ssn6 might retain partial activity. To ensure that those *ssn6* phenotypes did not result from the residual activity, we reisolated another mutant (CH603) deleted for all the TPR repeats (see *Experimental procedures*). As the reisolated strain

showed the same phenotypes as the other *ssn6/ssn6* mutant strains (CH403 and CH503), we could exclude the possibility that C-terminally truncated Ssn6 might affect the conditional pseudohyphal growth of *C. albicans.*

To establish the epistatic relationship between Ssn6 and Tup1, we constructed the *ssn6/ssn6 tup1/tup1* double mutant. In liquid YPD medium, the *ssn6/ssn6 tup1/tup1* double mutant was trapped in a stubby pseudohyphal growth pattern irrespective of a temperature change (Fig. 6A). In solid Spider medium, this double mutant formed wrinkled colonies with many invasive filaments similar to the *tup1/tup1* mutant (Fig. 6B). Nonetheless, microscopic observation revealed that such filaments were composed of thick rod-like cells nearly identical to the *ssn6* phenotype, although they were more elongated because of an increased number of attached cells (Fig. 6B). These results indicated that, as far as cell morphology was concerned, Ssn6 was epistatic to Tup1 at the higher temperature. Also, it is suggested that Tup1 plays a Ssn6-independent role in inhibiting filamentous growth, as the double mutant grew pseudohyphally at the lower temperature.

Overexpression of SSN6 *enhanced filamentous growth*

Based on our findings that the *ssn6/ssn6* mutant fails to develop true hyphae or extensively elongated filamentation, it is suggested that Ssn6 may act as an activator of filamentous growth. To test this suggestion, we placed *SSN6* under the control of the *PCK1* promoter (Leuker *et al*., 1997) and transformed a *PCK1*p-*SSN6* construct (pPSSN6) into wild-type *C. albicans*. Northern analysis revealed that the level of *SSN6* mRNA in this transformant was about 10.2-fold increased in SCAA medium compared with that of a control cell (data not shown). The *SSN6*-overexpressing cells showed enhanced filamentous growth in liquid SCAA medium at 28°C, conditions that did not induce morphogenesis in control cells carrying an empty vector (Fig. 7A). Some filaments produced by *SSN6* overexpression carried constriction at the side of

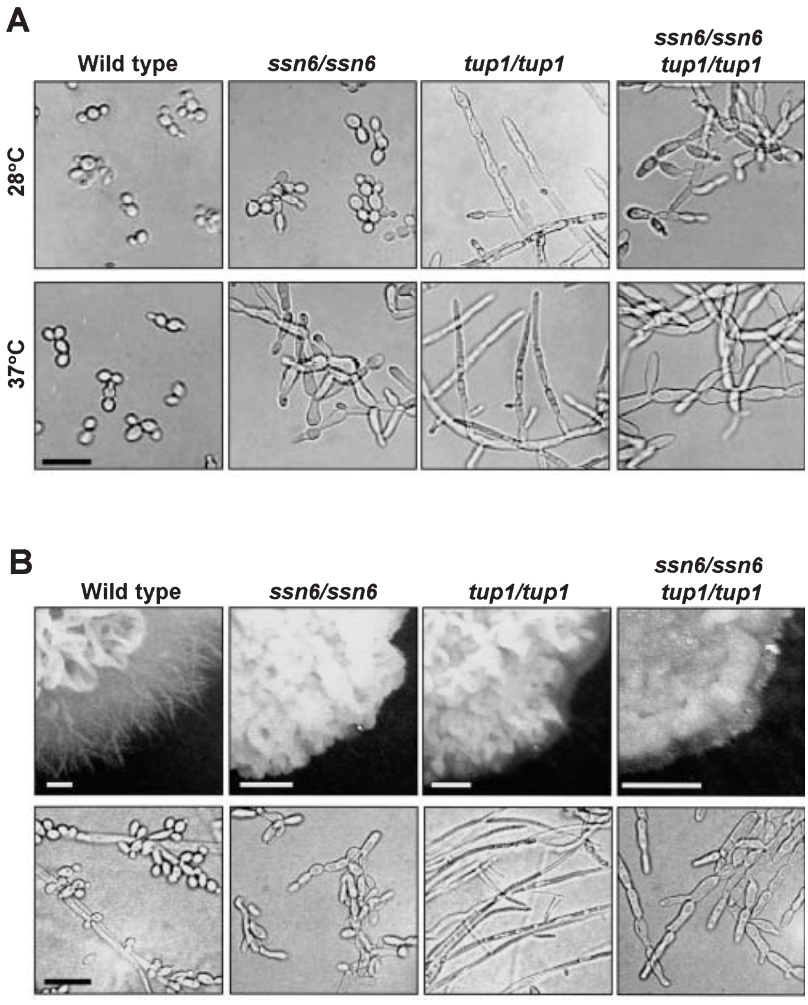

Fig. 6. Epistatic analysis of Ssn6 to Tup1.
A. The wild-type (SC5314), *ssn6/ssn6* (CH403), *tup1/tup1* (BCa2-10) and *ssn6/ssn6 tup1/tup1* (CH513) strains were grown in liquid YPD medium at 28°C or 37°C for 18 h (scale bar = 10 μm).
B. The indicated strains were also grown on solid Spider medium at 37°C for 7 days. The photographs are representative of the whole mounted microscopic plate (each scale bar = 5 mm). After the indicated strains were scraped from the interior of the agar, cell morphologies were observed (scale bar = 10 μm).

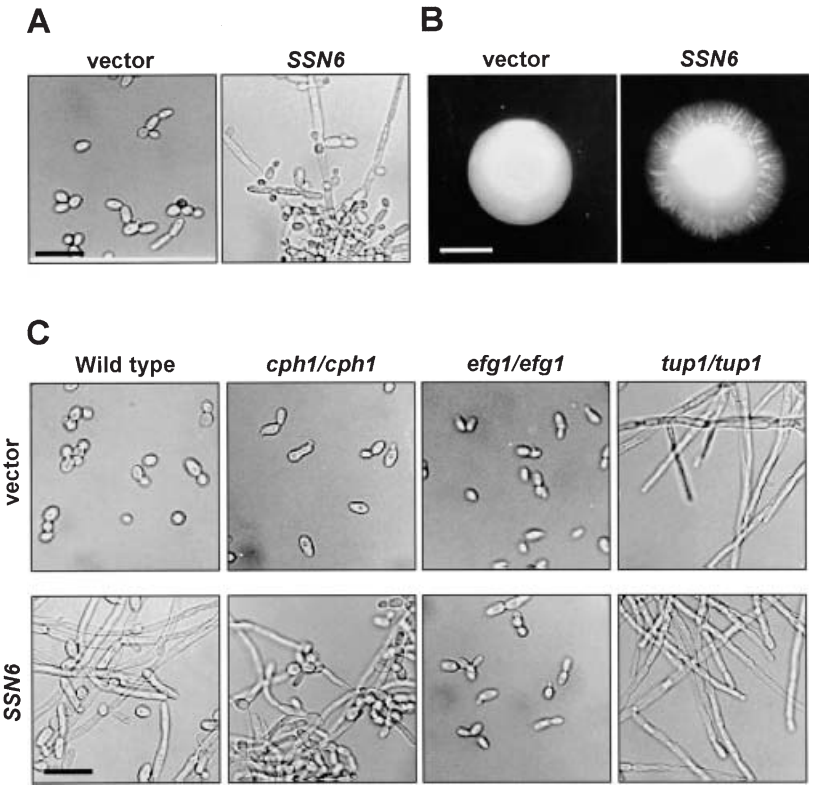

Fig. 7. Enhanced filamentous growth by *SSN6* overexpression. Wild-type strain CAI4 was transformed with control vector (pBI-1) or with the *SSN6*-overexpressing vector (pPSSN6). Cells were grown in liquid SCAA medium at 28°C for 18 h (scale bar = 10 μm) (A) or on solid YP1/4D medium for 5 days (scale bar = 5 mm) (B).
C. The wild-type (CAI4), *cph1/cph1* (JKC18), *efg1/efg1* (HLC67) or *tup1/tup1* (BCa2-9) strains carrying the empty (pBI-1) or *SSN6*-overexpressing vector (pPSSN6) were grown in SCAA at 28°C for 18 h (scale bar = 10 μm).

cell connections and generated by budding-like pseudohyphae when observed after staining with calcofluor white, but others appeared to be extremely elongated and contained parallel sides with abundant buds similar to true hyphae (data not shown). In solid SCAA medium, even control strains also produced extensive emerging filaments, and we tested the difference in filamentation on various solid media such as SD (synthetic dextrose), YP (1% yeast extract, 2% peptone), YP1/4D (YP + 0.5% glucose) and YPD. Although the *SSN6*-overexpressing cells were disposed to form more emerging filaments on all the solid media tested compared with the control, the obvious difference in filamentation was observed in YP1/4D solid medium. On this solid medium, the *SSN6*-overexpressing cells produced colonies with extensive emerging filaments in contrast to the smooth ones of the control cells (Fig. 7B). On the other hand, the transformation of pPSSN6 in the *ssn6/ssn6* mutant (CH404) resulted in cells that grew as yeast-like cells in YPD medium at 37°C, developed true hyphae in response to serum and displayed extensive filamentation on various solid media (data not shown). This indicated that *ssn6* phenotypes could be restored completely by episomal *SSN6* expression.

To address whether Ssn6-activated filamentous growth is associated with the Cph1- or Efg1-mediated signalling pathways, we next overexpressed *SSN6* in the *cph1/cph1* (JKC18) or *efg1/efg1* (HLC54) mutant strains. The overexpression of *SSN6* resulted in enhanced filamentous growth in wild-type (CAI4) or *cph1/cph1* strains, but not in the *efg1/efg1* mutant (Fig. 7C), indicating that Efg1 might be involved in the formation of filaments mediated by surplus Ssn6. On the other hand, the overexpression of *SSN6* could not rescue *tup1* phenotypes (Fig. 7C), or vice versa (Fig. 5A), suggesting that Ssn6 and Tup1 could not suppress each other's mutant phenotype.

Taken together, our findings suggest that Ssn6 may function as an activator as well as a repressor of filamentous growth in *C. albicans* and modulate morphological conversion between yeast and filaments through the direct or indirect interaction with Tup1- or Efg1-dependent pathways.

Impaired virulence by excess or deficient Ssn6 in mice

The importance of Ssn6 in the virulence of *C. albicans* is supported by experiments showing that *ssn6/ssn6* cells are avirulent in a mouse model system. All the mice injected with the *ssn6/ssn6* strain survived to the 30 day end-point, whereas other mice injected with either wild-type or revertant strains died by 15 days (Fig. 8A). Even injection with a 10-fold increase in *ssn6/ssn6* cells resulted in no fatalities, whereas the same number of wild-type or revertant cells resulted in death for the mice within

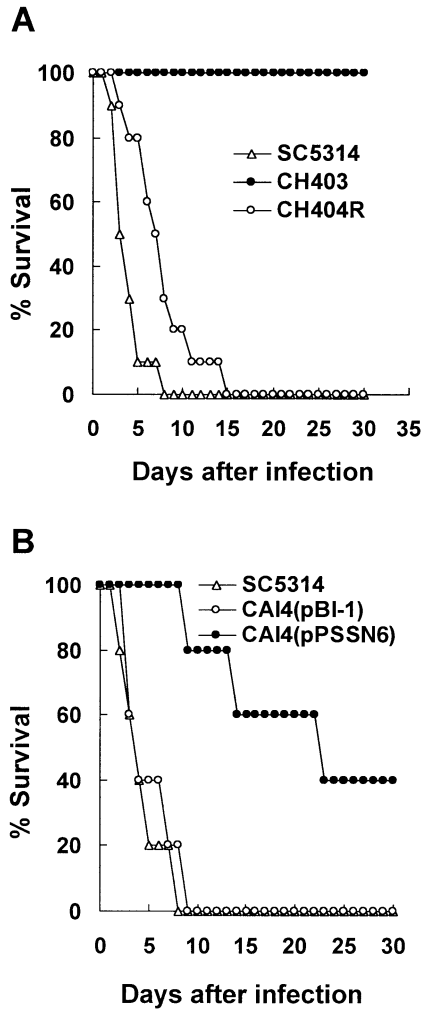

Fig. 8. Virulence assay of *C. albicans* in a mouse model. Survival of mice (10 for each group) was monitored after injection of *C. albicans* cells into the tail vein. Statistical analysis showed survival difference ($P < 0.001$) by the Wilcoxon rank sum test.
A. Avirulence by *ssn6* mutation. Wild-type SC5314, *ssn6/ssn6* mutant CH403 and revertant CH404R strains were injected intravenously into BALB/c mice.
B. Impaired virulence by *SSN6* overexpression. Wild-type SC5314, *SSN6*-overexpressing CAI4 (pPSSN6) and control CAI4 (pBI-1) strains were injected intravenously into BALB/c mice.

2 or 7 days respectively (data not shown). Furthermore, despite the promoted conversion from yeast to filamentous growth even in media that favours yeast-like growth, the *SSN6*-overexpressing *C. albicans* strain CAI4 (pPSSN6) attenuated virulence in mice compared with the wild-type SC5314 or control strain CAI4 (pBI-1) (Fig. 8B). As the *ssn6/ssn6* mutant grew poorly and aggregated somewhat in 10% serum agar or broth at the higher temperature of 37°C, the loss of its virulence seemed to be caused predominantly by a defect of growth in serum where temperature was constantly adjusted to 37°C. Unlike the *ssn6/ssn6* mutant, *SSN6*-overexpressing cells grew as well as the wild type in/on the various media including only 10% serum regardless of temperature. Thus, the impaired virulence of *SSN6*-overexpressing cells seems to result not from the growth defect but from other factors such as morphological or metabolic disturbances.

Discussion

Morphological phenotypes of the ssn6/ssn6 *mutant*

In the present study, the *C. albicans SSN6*, which could complement the phenotypes of *S. cerevisiae ssn6* mutation, was isolated and characterized. Disruption of both copies of *SSN6* led dramatically to cells that were trapped in stubby pseudohyphae and failed to develop true hyphae and extensive filamentation in/on almost all the media tested. If these pseudohyphae were an intermediate stage that would precede true hyphal development of wild type, the *ssn6* mutation would promote pseudohyphal growth in response to a strong true hyphal inducer, serum. However, the *ssn6/ssn6* mutant rarely, if ever, developed pseudohyphae in response to the medium containing only 10% serum (data not shown). Even in 10% serum + YPD medium at the lower temperature of 28°C (data not shown), it grew as yeast-like cells, whereas wild-type cells developed true hyphae or pseudohyphae. Thus, the pseudohyphae produced by *ssn6* mutation are unlikely to be intermediate stages of true hyphal development. Moreover, it has been demonstrated that pseudohyphae and true hyphae are qualitatively different forms and possibly formed by different pathways (Sudbery, 2001), although they were confused in the literature and often considered as a continuum of stages in a single pathway in *C. albicans* (Odds, 1985). According to this interpretation, *ssn6* mutation can lead to the derepression of the pseudohyphal growth pathway as well as the blockage of true hyphal development. The pseudohyphae produced by *ssn6* mutation were slightly different from those of wild type in that they were defective in filament elongation: the increased cell length results from the transition from thick rod-like into long tube-like cells and the increased number of attached cells. It is therefore suggested that Ssn6 might be required for the repression of stubby pseudohyphal growth, but also for the development of extremely elongated pseudohyphae and true hyphae.

Relationship between Ssn6 and Tup1 in C. albicans

In *S. cerevisiae*, Ssn6 forms a complex with Tup1, which, in turn, represses target genes via interaction with specific DNA-binding proteins. As *C. albicans SSN6* complemented an *S. cerevisiae ssn6* mutant, some effects of *ssn6* mutation, such as growth defect in non-glucose car-

bon sources and resistance against oxidants, were conserved in the two species. *C. albicans* Ssn6 has been suggested to function by a similar mechanism. However, considering that the *ssn6* mutant usually shared common phenotypes with the *tup1* mutant in *S. cerevisiae* (see *Introduction*), the effect of *ssn6* mutation in *C. albicans* was relatively weak compared with that of *tup1* mutation, with regard to pseudohyphal growth. For example, the pseudohyphal growth and expression of filament-specific genes in the *ssn6/ssn6* mutant are conditional depending upon elevated temperature (Figs 4 and 6A), whereas these are constitutive in the *tup1/tup1* mutant (Braun and Johnson, 1997; Braun *et al*., 2001). Moreover, deletion of *TUP1* in the *ssn6/ssn6* mutant abolished the conditional phenotypes and resulted in cells that were exclusively trapped in a stubby pseudohyphal growth pattern (Fig. 6). This different pseudohyphal growth pattern between *ssn6/ssn6* and *tup1/tup1* mutants suggests that Ssn6 may interact more weakly with Tup1 in *C. albicans* than in *S. cerevisiae* and that Ssn6 may also be needed to enhance full repression of pseudohyphal growth in contrast to the essential requirement of Tup1. Even in *S. cerevisiae*, Ssn6 is not absolutely essential for repression, but does enhance it, perhaps by helping to direct Tup1 to target promoters and to organize chromatin assembly (Keleher *et al*., 1992; Tzamarias and Struhl, 1994).

Nonetheless, Ssn6 may have some distinctive roles in the morphogenesis of *C. albicans.* First, Ssn6 is, perhaps, required for the elongation of pseudohyphae, as the *ssn6/ssn6* mutant could not develop from thick rod-like (*ssn6* phenotype) into long tube-like cells (*tup1* phenotype) even in the absence of Tup1. Secondly, as discussed below, Ssn6 may act as an activator as well as a repressor of filamentous growth in *C. albicans*, unlike Tup1, considering that overexpression of *SSN6* led to enhanced filamentous growth, whereas that of *TUP1* did not affect cell morphology.

Overexpression of SSN6 *led to enhanced filamentous growth*

Very interestingly, Ssn6 appeared to activate as well as to repress filamentous growth in *C. albicans*, as the *ssn6/ssn6* mutant failed to develop true hyphal development and extensive filamentation (Fig. 3), and overexpression of *SSN6* led to enhanced filamentous growth (Fig. 7). It is a surprising finding that disruption and overexpression of *SSN6* both led to the promotion of filamentous growth. There are two plausible explanations for this phenomenon. First, it is conceivable that the overexpressed Ssn6 has a 'squelching' mechanism (or a gain of function). In other words, surplus Ssn6 sequesters the repressor complex components (Tup1 or specific DNA-binding proteins), then inactivates either of them, and thereby promotes filamentous growth. Secondly, the overexpressed Ssn6 could, according to the literature, activate a morphogenetic process related to the filamentous growth pathway. Under certain circumstances, even *S. cerevisiae* Ssn6 also acts as a co-activator in the transcriptional control of several genes as well as a co-repressor. For example, it is required for the activation of cytochrome *c* gene (*CYC1*) transcription (Zhang and Guarente, 1994), the maximal induction of the invertase gene (*SUC2*) (Ozcan *et al*., 1997), and the expression of a peroxisomal citrate synthase gene (*CIT2*) by mitochondrial dysfunction (Conlan *et al*., 1999). Most recently, it has been reported that, during transcriptional induction in response to stress, Ssn6-Tup1 does not release from target promoters but rather remains bound to them and subsequently recruits SAGA histone acetylase, which promotes RNA polymerase II binding and transcriptional activation (Papamichos-Chronakis *et al*., 2002; Proft and Struhl, 2002). In addition, the finding that overexpression of *SSN6* did not repress the formation of true hyphae by serum is in agreement with the possibility that Ssn6 can be an activator of true hyphal growth.

Our results show that Ssn6 appears to function in both positive and negative regulation of filamentous growth in *C. albicans*. It has already been demonstrated that some factors play a dual function as an activator as well as a repressor of filamentous growth in *C. albicans*. For example, reduced *EFG1* expression and its overexpression both stimulate filamentous growth of a different type, although the *efg1/efg1* mutant or *EFG1*-overexpressing cells formed rod-like elongated or extremely extended pseudohyphae respectively (Lo *et al*., 1997; Stoldt *et al*., 1997). Also, it has reported that Efg1 acts as a repressor of filamentous growth under embedded or microaerophilic conditions (Ernst, 2000; Giusani *et al*., 2002). Furthermore, a *C. albicans* strain lacking of Rfg1, a close relative of *S. cerevisiae* Rox1, showed enhanced or reduced filamentation depending upon environmental conditions (Kadosh and Johnson, 2001).

In *S. cerevisiae*, 3% of the total genes are reported to be controlled by Ssn6-Tup1 (DeRisi *et al*., 1997; Smith and Johnson, 2000). Moreover, the recent transcription profiling data on Tup1-, Mig1- or Nrg1-regulated *C. albicans* genes demonstrate that these factors have pleiotropic effects on the regulation of carbon metabolism, stress response and cellular morphogenesis (Murad *et al*., 2001b). Therefore, we cannot entirely preclude the possibility that filamentous growth by disruption or overexpression of *SSN6* may be mediated indirectly through disturbance of nutrient metabolism, stress response and genetic machinery abnormality.

Impaired virulence by deficient and surplus Ssn6

The reversible morphological conversion between yeasts and filamentous forms is believed to be important for virulence, because *C. albicans* strains locked in either yeasts (Lo *et al*., 1997) or filamentous forms (Braun and Johnson, 1997) are avirulent in mice. Therefore, the result that defective or promoted ability to convert cellular morphology impairs disease development suggests that wild-type *C. albicans* presumably optimizes timely cellular morphology to promote virulence in response to external stimuli. However, the lack of virulence by *ssn6* mutation is unlikely to result from only the defective morphological conversion, as the *ssn6/ssn6* mutant failed to grow in serum at 37°C. Accordingly, the loss of virulence in the *ssn6/ssn6* mutant mainly appeared to result from poor growth within host serum, where the temperature was constantly adjusted to 37°C. Moreover, metabolic defects of this mutant in non-fermentable carbon sources (acetate or lactate) could be responsible for impaired virulence. Genome-wide analyses of the functions of Tup1, Mig1 and Nrg1 in *C. albicans*-regulated genes also provided important clues about the global roles in cellular morphology, carbon metabolism, stress responses and virulence (Murad *et al*., 2001b). It is accordingly conceivable that the surplus or deficient Ssn6 may impair the pathogenicity of *C. albicans* through their pleiotropic effects as well as morphological defects.

In conclusion, *C. albicans* can undergo morphological conversion between yeast and filamentous forms in response to a wide variety of environmental stimuli, including serum, neutral pH, elevated temperature, nutrient starvation and limited oxygen tension (Odds, 1985; Ernst, 2000). As the changes in *SSN6* expression led to loss of the ability to optimize cellular morphology and virulence, Ssn6 seems to be a key factor in morphological conversion and virulence. Furthermore, our findings that the overexpression and disruption of *SSN6* impair pathogenicity of *C. albicans* will provide a detailed analysis of virulence determinants as well as the precise identification of putative drug targets.

Experimental procedures

Culture media

Escherichia coli DH5α was used for the maintenance of plasmids. *E. coli* XL1-Blue MRA (P2) was used in the screening of a *C. albicans* genomic library contained in λEMBL3. *C. albicans* and *S. cerevisiae* strains and plasmids used in this study are listed in Tables 2, 3 and 4 respectively. *E. coli* was grown at 37°C in Luria–Bertani medium and in NZY medium (0.5% NaCl, 0.2% $MgSO_4.7H_2O$, 0.5% yeast extract, 1% NZ amine) when proliferating bacteriophage. For routine growth of *C. albicans* or *S. cerevisiae* cells, YPD medium was used. Cells containing plasmids or disrupted genes were cultured in SD medium consisting of 0.67% yeast nitrogen base without amino acids (Difco), 2% glucose and appropriate supplements (Kaiser *et al*., 1994). *C. albicans* strains overexpressing *SSN6* were grown in SCAA medium (0.67% yeast nitrogen base without amino acids, 2% casamino acids) to induce *PCK1* promoter. Corn meal agar medium was composed of 1.7% corn meal agar and 0.33% Tween 80. Ura^- auxotrophs were selected on minimal defined medium supplemented with 625 mg of 5-fluoroorotic acid and 25 mg of uridine per litre (FOA medium). Before selection, cells were plated on YPD medium and incubated for 48 h. Individual colonies were taken from the plate and suspended in distilled water. A portion of the suspension was spread on FOA

Table 2. *C. albicans* strains used in this study.

Strain	Genotype	Sources
ATCC10231	Wild type	ATCC[a]
SC5314	Wild type	Fonzi and Irwin (1993)
CAI4	*ura3::1 imm434/ura3::1 imm434*	Fonzi and Irwin (1993)
BCa2-9	*ura3::1 imm434/ura3::1 imm434 tup1::hisG/tup1::hisG*	Braun and Johnson (1997)
BCa2-10	*ura3::1 imm434/ura3::1 imm434 tup1::hisG/tup1::hisG-URA3-hisG*	Braun and Johnson (1997)
JKC18	*ura3::1 imm434/ura3::1 imm434 cph1::hisG/cph1::hisG*	Lo *et al.* (1997)
HLC67	*ura3::1 imm434/ura3::1 imm434 efg1::hisG/efg1::hisG*	Lo *et al.* (1997)
CH401	*ura3::1 imm434/ura3::1 imm434 ssn6::hisG-URA3-hisG/SSN6*	This study
CH402	*ura3::1 imm434/ura3::1 imm434 ssn6::hisG/SSN6*	This study
CH403	*ura3::1 imm434/ura3::1 imm434 ssn6::hisG/ssn6::hisG-URA3-hisG*	This study
CH404	*ura3::1 imm434/ura3::1 imm434 ssn6::hisG/ssn6::hisG*	This study
CH404R	As CH404, but *ssn6:: SSN6-URA3*-pBluescript KS+	This study
CH503	*ura3::1 imm434/ura3::1 imm434 ssn6::hph/ssn6::hph-URA3-hph*	This study
CH504	*ura3::1 imm434/ura3::1 imm434 ssn6::hph/ssn6::hph*	This study
CH511	As CH504, *tup1::hisG-URA3-hisG/TUP1*	This study
CH512	As CH504, *tup1::hisG/TUP1*	This study
CH513	As CH504, *tup1::hisG/tup1::hisG-URA3-hisG*	This study
CH514	As CH504, *tup1::hisG/tup1::hisG*	This study
CH603	The same genotype as CH403, but a large deletion of *SSN6* coding region	This study

a. American Type Culture Collection.

Table 3. *S. cerevisiae* strains used in this study.

Strain	Genotype	Sources
MCY829	*MATα hisΔ20 lys2-801 ura3-52*	Treitel and Carlson (1995)
MCY829-YEp	MCY829, but YEp352	This study
MCY1974	*MATα hisΔ20 lys2-801 ura3-52 ade2-101 trp1Δ ssn6Δ9*	Treitel and Carlson (1995)
MCY1974-YEp	As MCY1974, but YEp352	This study
MCY1974-SSN6	As MCY1974, but YEp352-SSN6	This study
SH4038	*ura3-52 his4-539 lys2-801 leu2-3112 trp1 pho3-1 pho5-1 ssn6::URA3*	Mukai *et al.* (1999)
SH4038SP	As SH4038, but *leu2::LEU2-STE6-PHO5* in SHB2019	This study
SH4038SP-pRS	As SH4038SP, but pRS424	This study
SH4038SP-SSN6	As SH4038SP, but pRS424-SSN6	This study

medium and incubated for 3–4 days. To induce hyphae, *C. albicans* cells were grown previously at 28°C in YPD to the exponential growth phase and then diluted ($A_{600} = 0.1$) into YPD, 10% fetal calf serum, Lee's and RPMI-1640 liquid media at 37°C. Cell morphology was photographed using a Zeiss MC200 camera under light microscope with 40× objectives.

Cloning and sequencing

Using the probe based on a sequence trace of *SSN6* from the Stanford *C. albicans* sequencing project (http://www-sequence.stanford.edu/group/candida), the following oligonucleotide primers were synthesized: pS1, 5′-ATCATATC CATCTACACCAG-3′; and pS2, 5′-GATCATGAGTCCAATTG AAC-3′. A polymerase chain reaction (PCR) amplification was carried out for 30 cycles under the following conditions; denaturation at 94°C for 3 min, annealing at 42°C for 1 min and extension at 72°C for 1 min. The resulting 248 bp fragment was labelled with digoxigenin (DIG; Roche Molecular Biochemicals) and used as a probe to screen the λEMBL3 genomic library (Hwang *et al.*, 1999). Three positive clones were selected, and the common 5.3 kb *Eco*RI–*Eco*RV fragment giving a positive signal was isolated and cloned into pBluescript-II KS+ (Stratagene) at the *Eco*RI–*Eco*RV site, yielding pSSN6. Both strands of the cloned DNA were sequenced. The nucleotide sequence of *SSN6* from *C. albicans* ATCC 10231 has been deposited in the GenBank database under accession number AF170083.

Northern analysis

Strain SC5314 was grown in YPD to saturation and then diluted to a cell density (A_{600}) of ≈0.5 in YPD supplemented with 10% serum. At the indicated times, total RNA was prepared using hot phenol. RNA (40 μg) from each sample was separated on a 1% agarose gel containing 0.22 M formaldehyde and blotted onto Magna nylon membrane (Osmonics). The coding region of *SSN6* was PCR amplified and labelled with [α-^{32}P]-dATP. All blots were prehybridized for 1 h, hybridized for 2 h and washed twice for 20 min at 68°C.

Complementation of S. cerevisiae ssn6 *mutation*

To construct a vector complementing the *ssn6* mutation of *S. cerevisiae*, the *Eco*RI–*Eco*RV fragment of pSSN6 was inserted into the *Eco*RI–*Sma*I-digested YEp352 (Hill *et al.*, 1986), yielding plasmid YEp352-SSN6. Plasmids YEp352 and YEp352-SSN6 were used for transformation in *S. cerevisiae* parental strain MCY829 or *ssn6* mutant strain MCY1974 (Treitel and Carlson, 1995). To assess Ssn6 function under the control of mating type, *S. cerevisiae ssn6* mutant SH4038 was integrated at the *Afl*II site within *leu2* using the plasmid harbouring *STE6-PHO5* (Mukai *et al.*, 1999), which had an acid phosphatase reporter gene. The *S. cerevisiae ssn6* mutant strain SH4038 harbouring *STE6-PHO5* (SH4038SP) was transformed with the plasmid pRS424 (Sikorski and Hieter, 1989), and pRS424-SSN6 was produced by insertion of the *Eco*RI–*Xho*I fragment of pSSN6 into *Eco*RI–*Xho*I-digested pRS424.

Gene disruption and overexpression

Disruption of *SSN6* in *C. albicans* was carried out as described by Fonzi and Irwin (1993). First, a 2.2 kb *Pst*I-digested DNA fragment within *SSN6* was inserted into the

Table 4. Plasmids used in this study.

Plasmid	Description	Reference or source
pSSN6	Genomic *SSN6* fragment in pBluescript KS+	This study
p5921	*hisG-URA3-hisG* cassette in pUC18	Fonzi and Irwin (1993)
pQF18	*hph-URA3-hph* cassette in pBluescript KS+	Feng *et al.* (1999)
pBI-1	*PCK1* promoter in *URA3*-marked CaARS vector	Stoldt *et al.* (1997)
pRC2312P-H	*PCK1* promoter::*EFG1* in pBI-1	Stoldt *et al.* (1997)
pPSSN6	*PCK1* promoter::*SSN6* in pBI-1	This study
YPB1-ADHpL	*ADH1* promoter and terminator in *URA3*-marked 2 μm vector	Csank *et al.* (1998)
pLJ19	*ADH1* promoter::*CPH1* in YPB1-ADHpL	Csank *et al.* (1998)
pATUP1	*ADH1* promoter::*TUP1* in YPB1-ADHpL	This study

pGEM-5Zf(+) vector (Promega). Then, the disruption construct was created by inserting a *Bam*HI–*Bgl*II-digested *hisG-URA3-hisG* cassette from p5921 (Fonzi and Irwin, 1993) at the two *Bam*HI sites to remove 0.7 kb from the *SSN6* coding region of *C. albicans*. The construct yielded was linearized with *Apa*I–*Sac*I, transformed into CAI4 (a Ura$^-$ derivative of wild-type strain SC5314; Fonzi and Irwin, 1993) and selected by uracil prototrophy. The integration of these cassettes into the *SSN6* locus was verified by Southern analysis. Ura$^-$ derivatives of the heterozygous disruptants were selected on FOA medium. This procedure was repeated once more to generate homozygous a *ssn6/ssn6* mutant strain (CH403). For reintegration of the *SSN6* into the genome, an *Xba*I–*Sca*I-digested *URA3* fragment from pURA3 (Huh *et al*., 2001) was inserted into the *Xba*I–*Eco*RV sites of pSSN6. The pSSN6-URA3 was linearized at the unique *Afl*II site of the coding region of *SSN6* and used to integrate into the genomic *SSN6* locus in the Ura$^-$ *ssn6/ssn6* strain (CH404). The targeted reintegration was confirmed by genomic PCR (data not shown). For the large deletion of the *SSN6* coding region, first, a 5.3 kb *Nco*I–*Eco*RV-digested DNA fragment of pSSN6 was inserted into inserted into the pGEM-5Zf(+) vector (Promega). Another disruption construct was created by inserting a *hisG-URA3-hisG* cassette (Fonzi and Irwin, 1993) at *Pst*I–*Kpn*I sites to remove 2.7 kb of *SSN6* coding region (amino acid residues 61 and 958). The construct was also used to generate a homozygous *ssn6/ssn6* (CH603) mutant strain deleted for all the TPR regions, as described above.

To discover the relationship between Ssn6 and Tup1, we tried to disrupt *TUP1* in the *ssn6/ssn6* (CH404) strain by performing transformation with a plasmid *hisG-URA3-hisG* cassette. However, it turned out to be very difficult because the plasmid integrated at high frequency into the *hisG* region flanked by disrupted *SSN6* rather than *TUP1*. To avoid this problem, we adopted another disruption cassette, *hph-URA3-hph*, in which the *hisG* segment was replaced by the *E. coli* hygromycin gene (Feng *et al*., 1999). Moreover, to facilitate the disruption of both copies of *SSN6* in each strain, we used homologous recombination in two steps by the *hph-URA3-hph* cassette, which exploited an idea described by Pérez-Martín *et al*. (1999) to allow the cassette to be cloned in two opposite orientations within the target *SSN6* gene. The *Bam*HI-digested fragment of *hph-URA3-hph* from pQF86 (Feng *et al*., 1999) was inserted into the *Bam*HI-cut pUC18 at two different orientations, yielding pQF181 and pQF182. The *hph-URA3-hph* cassettes generated from the *Hin*dIII–*Kpn*I-digested pQF181 or pQF182 were inserted at the *Hin*dIII–*Kpn*I sites of the *SSN6* coding region respectively. These constructs were used to make another *ssn6/ssn6* mutant (CH504) strain, and the disruption of *SSN6* was confirmed by PCR. Disruption of *TUP1* in this CH504 strain was subsequently performed using a *hisG-URA3-hiG* cassette, which was inserted into the *Bgl*II–*Sal*I sites of its coding region, as described above. The resulting disruption of *TUP1* in the CH504 strain was confirmed by Southern analysis (data not shown).

To overexpress *SSN6* in *C. albicans*, pBI-1 (Stoldt *et al*., 1997) vector containing the *C. albicans PCK1* promoter was used. A *Bgl*II site was placed upstream of the putative *SSN6* ATG start codon by PCR amplification of a 0.5 kb *Bgl*II–*Afl*II fragment and inserted into pSSN6 to generate pSSN6(Bg). The *SSN6*-overexpressing vector (pPSSN6) was constructed by inserting the fragment of *Bgl*II–*Eco*RV from pSSN6(Bg) into the same sites downstream of the *PCK1* promoter within pBI-1. The resultant plasmid, pPSSN6, was transformed into each isogenic Ura$^-$ strain CAI4, CH404, BCa2–9, JKC18 or HLC67.

To overexpress *TUP1* in *C. albicans*, the coding region was amplified with *Pfu* polymerase (Promega). The primers used for synthesis of *TUP1* were 5′-CAGGATCCATGTCCATG TATCCCC-3′ and 5′-CTCTCGAGTTATTTTTTGGTCCAT-3′. The amplification product was digested with *Bam*HI–*Xho*I and then cloned into the *Bgl*II–*Xho*I sites of plasmid pYPB1-ADHpL (Csank *et al*., 1998). The resultant plasmid, pATUP1, was transformed into wild type (CAI4) or the *ssn6/ssn6* (CH404) strain, and overexpression of Tup1 was confirmed by Western analysis using polyclonal α-Tup1. For the heterologous expression of Tup1 in *E. coli*, the PCR-amplified sequence using the following primers (5′-CATATGTCCATG TATCCCCA-3′ and 5′-GGATCCTTATTTTTTGGTCC-3′) was cloned into pET-3a (Novagen), and then the plasmid yielded was transformed into BL21(DE3)pLysS cells. After gel electrophoresis of cell extracts, the Tup1 band was cut out from the gel and injected into ICR female mice to raise polyclonal antibody against Tup1.

Virulence study

BALB/c (female) mice weighing between 17 and 20 g were used to test the virulence of different strains. Two experiments were initiated by growing the *Candida* strains on YPD plates for 48 h at 28°C, suspending the cells in phosphate-buffered saline (pH 7.5) and adjusting them to the desired concentration after the measurement of cell density ($A_{600} = 0.5$). Approximately 0.1 ml of *Candida* cell suspension (1×10^7 or 1×10^8 cells ml^{-1}) was injected into mice.

Other methods

For enzyme assays, yeast cells were grown in YPD medium up to early exponential phase ($A_{600} = 1$), harvested by centrifugation at 12 000 r.p.m. for 5 min and then homogenized with a Mini Bead-Beater (Biospec Products) for 90 s in 1 ml of ice-cold 50 mM potassium phosphate buffer (pH 7.8) containing 0.1 mM EDTA. To measure cell density, 10 mM EDTA was added to disperse the cell clumps in flocculent cultures. Acid phosphatase and invertase activities were determined according to the methods proposed by To-E *et al*. (1973) and Goldstein and Lampen (1975) respectively.

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