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Roles of Zinc-responsive Transcription Factor Csr1 in Filamentous Growth of the Pathogenic Yeast *Candida albicans*

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In the fungal pathogen *Candida albicans*, the yeast-to-hyphal transition occurs in response to a broad range of environmental stimuli and is considered to be a major virulence factor. To address whether the zinc homeostasis affects the growth or pathogenicity of *C. albicans*, we functionally characterized the zinc-finger protein Csr1 during filamentation. The deduced amino acid sequence of Csr1 showed a 49% similarity to the zinc-specific transcription factor, Zap1 of *Saccharomyces cerevisiae*. Sequential disruptions of *CSR1* were carried out in diploid *C. albicans*. The *csr1/csr1* mutant strain showed severe growth defects under zinc-limited growth conditions and the filamentation defect under hypha-inducing media. The colony morphology and the germ-tube formation were significantly affected by the *csr1* mutation. The expression of the hyphae-specific gene *HWP1* was also impaired in *csr1/csr1* cells. The *C. albicans* homologs of *ZRT1* and *ZRT2*, which are zinc-transporter genes in *S. cerevisiae*, were isolated. High-copy number plasmids of these genes suppressed the filamentation defect of the *csr1/csr1* mutant strain. We propose that the filamentation phenotype of *C. albicans* is closely associated with the zinc homeostasis in the cells and that Csr1 plays a critical role in this regulation.

Keywords: Fungal pathogen, *Candida albicans*, pathogenicity, zinc-finger domain, Zap1, Csr1, zinc transporter, filamentation

Candida albicans is the most prevalent fungal pathogen of humans, which causes mucosal infections in relatively healthy individuals and also life-threatening systemic infections in premature infants, surgical patients, chemotherapy patients, and other patients with weakened immune systems [4, 20]. Mortality from systemic infections approaches 30% despite appropriate therapy with the available antifungal agents [11, 20]. *C. albicans* is a polymorphic yeast that undergoes reversible morphogenetic transitions among budding, pseudohyphal, and hyphal growth forms [15, 19, 26]. Its ability to switch between yeast and hyphal growth forms is directly correlated with its virulence. The yeast-to-hyphal transition occurs in response to a broad range of environmental stimuli, such as serum availability, the presence of specific compounds (such as *N*-acetylglucosamine), temperature (37°C), or pH.

Several signaling pathways that include a Cph1-mediated mitogen-activated protein kinase and an Efg1-mediated cyclic AMP/protein kinase A have been known to participate in the regulation of the morphological transitions [15, 16]. These multiple pathways in conjunction with the pathway-specific transcription factors regulate the expressions of hypha-specific genes, such as *ECE1*, *HWP1*, *HYR1*, *ALS3*, *ALS8*, *RBT1*, and *RBT4* [4]. Many of these hypha-specific genes encode either cell wall or secreted proteins.

Zinc is an essential nutrient in the cell, functioning as a structural component of the zinc-finger motifs found in many transcription factors and as a catalytic cofactor for cellular enzymes such as RNA polymerase [3, 14, 21]. As with other metal ions, zinc homeostasis is critical for the cell growth and viability. In a previous report, the concentration of the zinc ion in the culture media affected the hyphal formation of *C. albicans* [2]. The zinc levels in the body fluid of infected individuals were reported to be related to the growth and the pathogenicity of *C. albicans*. The Zap1 transcriptional activator of *Saccharomyces cerevisiae* is responsible for the regulation of the zinc transporter genes *ZRT1*, *ZRT2*, and *ZRT3* [21]. In addition to the zinc transporter genes, Zap1 upregulates the expression of its own promoter *via* a positive autoregulatory mechanism [27]. DNA microarray analysis suggested that Zap1 controls the expression of as many as 42 other genes in response to zinc status [17].

In a previous study, two genes of *C. albicans*, *CHR1* (*Candida* Homolog of *ROK1*) and *CSR1* (*Candida* Suppressor of *rok1*), were isolated as a high copy-number plasmid suppressor of the *rok1* null mutation [12]. The *ROK1* gene, encoding a DEAD-box RNA helicase, is shown to be involved in cell cycle progression [24]. Chr1 shows 54% identity in amino acid sequences with Rok1

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and is predicted to be a *C. albicans* DEAD-box RNA helicase, whereas the putative gene product of *CSR1* does not show any similarities in amino acid sequence to Rok1. Csr1 contains putative zinc-finger domains.

In this work, we performed the functional characterizations of *CSR1* in *C. albicans*. Amino acid sequence alignment showed that Csr1 is a homolog of zinc-specific transcription factor Zap1. Sequential gene disruptions of *CSR1* were carried out in a *C. albicans* diploid strain and the essential roles of *CSR1* in the *C. albicans* filamentation were addressed. We propose a close association of the filamentation phenotype with the zinc homeostasis in *C. albicans*.

MATERIALS AND METHODS

Strains, Plasmids, and Growth Conditions

All strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strain DH5α was used to amplify plasmid DNA. Yeast strains were grown in YEPD (1% yeast extract, 2% peptone, 2% dextrose) or Synthetic Complete (SC, 0.67% yeast nitrogen base w/o amino acid, 2% dextrose, all amino acid required) media at 30°C. For URA3 pop-outs, cells were streaked on 5-FOA media (0.67% yeast nitrogen base w/o amino acid, 2% dextrose, 0.005% uridine, 0.1% 5-fluoroorotic acid).

To test the viability of the mutants in zinc-limiting media, cells were pregrown overnight in YEPD media and then diluted into YNB (0.67% yeast nitrogen base, 25 μg/ml uridine, 2% agar, 2% dextrose), low zinc (YNB, 1 mM EDTA, 100 μM $ZnCl_2$), and high zinc (YNB, 1 mM EDTA, 1 mM $ZnCl_2$) media. Plates were incubated at 30°C for 3 days.

Hyphal Growth Test in *C. albicans*

To test the filamentation phenotype of *C. albicans*, cells were plated (about 120 cells per plate) on solid YEPD containing 10% fetal bovine serum, solid Spider medium (1% mannitol, 1% nutrient broth, 2% agar, 0.2% K_2HPO_4, pH 7.2), or solid modified Lee's medium (0.67% yeast nitrogen base w/o amino acid, auxotrophic requirement 50 μg/ml, 2% mannitol, 5% serum, 2% agar, pH 7.2) and then incubated at 37°C for 2–7 days. To induce hyphal formation in liquid media, cells were pregrown overnight in YEPD media at 30°C, diluted (1×10^6/ml) into the indicated inducing medium, and incubated at 37°C.

Transformation and DNA Manipulation Technique

C. albicans transformation was performed by the lithium acetate method using 50 μg of salmon sperm carrier DNA [1]. Standard molecular biological techniques were performed to construct the plasmid [22]. Yeast genomic DNA was prepared by the rapid isolation method and used as a template for PCR amplification [1]. Restriction enzymes were purchased from Boerhinger Mannheim (BM), New England Biolab (NEB), and MBI Fermentas.

Sequence Analysis of *C. albicans CSR1*, *ZRT1*, and *ZRT2*

The complete genomic DNA sequences of *CSR1*, *ZRT1*, and *ZRT2* were searched in the *C. albicans* genome database at Stanford University (http://candida.stanford.edu/group/candida). Sequence analysis was processed by the computer-based World Wide Web searches of nucleotide and amino acid databases (http://www.ncbi.nlm.nih.gov/BLAST/) and multiple alignment program.

Disruption of *C. albicans CSR1*

The *CSR1* disruption was carried out by using a modified Ura-blaster method [10]. For the sequential disruption of two copies of *CSR1*, two plasmids (pcsr1::hisG and pcsr1::hph) were constructed. Two fragments of *CSR1* were obtained by PCR using four primers, CadS1 (5'CCATCGATATCTTGAGTGTTGCGATGATCC-3'), CadS2 (5'GAAGATCTCTGTTGCTCCTGTTGCTCATG-3'), CadS3 (5'GA-AGATCTCGGGACACATCGTAAAAGAATAC-3'), and CadS4 (5'AGGAGCTCGATATCTACTTGGTGCATATATACAGGCTG-3'). These fragments were ligated into the pRS316 vector, generating pRS316-DS1. The BglII-BamHI fragment of the *hisG-CaURA3-hisG* cassette from pCUB-6 [10] was inserted at the BglII site of pRS316DS, generating plasmid pcsr1::hisG. To construct the second

Table 1. Strains and plasmids used in this study.

Strain or plasmid	Genotype	Source or reference
C. albicans		
SC5314	Wild type	G. R. Fink
CAI4	*ura3::imm434/ura3::imm434*	G. R. Fink
JKC50	*ura3/ura3, csr1::hisG-CaURA3-hisG/CSR1*	This study
JKC51	*ura3/ura3, csr1::hisG/CSR1*	This study
JKC52	*ura3/ura3, csr1::hisG/csr1::hph-CaURA3-hph*	This study
JKC53	*ura3/ura3, csr1::hph/csr1::hisG*	This study
Plasmids		
pRS316	*CEN ARS URA3*	[20]
pRS316DS1	*CSR1*(+247~+795, +2,000~+2,600)*CEN ARS URA3*	This study
pRS316DS2	*CSR1*(+247~+795, +1,227~+1,847)*CEN ARS URA3*	This study
pcsr1::hisG	*csr1::hisG::CaURA3::hisG::csr1 CEN ARS URA3*	This study
pcsr1::hph	*csr1::hph::CaURA3::hph::csr1 CEN ARS URA3*	This study
pRC18	*URA3*-marked *CARS2*-vector	[22]
pRC18-CSR1	pRC18 containing *CaCSR1*	This study
pRC18-ZRT1	pRC18 containing *CaZRT1*	This study
pRC18-ZRT2	pRC18 containing *CaZRT2*	This study

disruption plasmid, primers CadS1 (5'CCATCGATATCTTGAGTGT-TGCGATGATCC-3'), CadS2 (5'GAAGATCTCTGTTGCTCCTGTT-GCTCATG-3'), CSR1 2DT-F (5'-GGGAGATCTCTTCGGTGAAA-ATCGTGCCC-3'), and CSR1 2DT-R (5'-GGGGAGCTCTACTTCC-CCCCTATACCGAG-3') were used. The PCR fragments were ligated into the pRS316 vector, generating pRS316-DS2. The BglII fragment of *hph-CaURA3-hph* from pQF86 [9] was ligated into pRS316-DS2, generating pcsr1::hph.

For disruption of *CSR1*, a *DrdI-Eco*72I fragment of pcsr1::hisG was used for transformation of strain CAI4. One copy disruption strain, JK360 (*CSR1/csr1::hisG-CaURA3-hisG*), was confirmed by PCR and was patched on 5-FOA plates for selection of URA3 pop-out colonies. The resulting strain JK361 (*CSR1/csr1::hisG*) was used for a second round of gene disruption. A linear ClaI-SacI fragment of pcsr1::hph was transformed into JK361 to generate strain JK362 (*csr1::hisG/csr1::hph-CaURA3-hph*). JK363 (*csr1::hisG/csr1::hph*) was constructed by URA3 pop-out on 5-FOA [13]. The confirmation of the *CSR1* disruption was carried out by both PCR analysis (Fig. 2) and Sourthern blot (data not shown).

Northern Blot Analysis

Total RNA was prepared as previously described [8]. Twenty μg of total yeast RNA was fractionated by electrophoresis through 1.0% formaldehyde gel and was subsequently transferred to a Nytran membrane (Hoefer). Blottings were performed as previously described [22]. DNA probes were generated by PCR using the following primers: CaACT1 (CaACT1-NF: 5'-ACCGAAGCTCCAATGAAT-CCA-3'; CaACT1-NR: 5'-GGATGGACCAGATTCGTCGTA-3'); HWP1 (HWP1-NF: 5'-GCTCAACTTATTGCTATCGC-3'; HWP1-NR: 5'-CAGGCTGATCAGGTTGAG-3'). Labeling of the probes was done with the Rediprime II, random primer labeling system from Amersham Biosciences.

RESULTS

Csr1 is a *C. albicans* Homolog of Zinc-responsive Transcription Factor Zap1

The *CSR1* gene contains an open reading frame of 1,836 bp, encoding a novel protein with five zinc-finger motifs [12]. The deduced amino acid sequence of Csr1 showed a high similarity (34% identity and 49% positivity) to the zinc-specific transcription factor, Zap1 of *S. cerevisiae*. The Zap1 protein contains a large number of potential zinc-binding residues at the C-terminus, which constitute seven zinc-fingers of C_2H_2 type (Fig. 1) [27]. The N-terminal region of Zap1p has two acidic activation domains and is rich in histidine and cysteine residues (about 12%) [3, 27]. The Csr1 protein has five zinc-finger motifs in the central (321–345) and C-terminal regions (499–524, 530–552, 558–582, 586–608) (Fig. 1). The sequence alignment in BLAST search showed that the highest similarity between Csr1 and Zap1 is found in these zinc-finger regions.

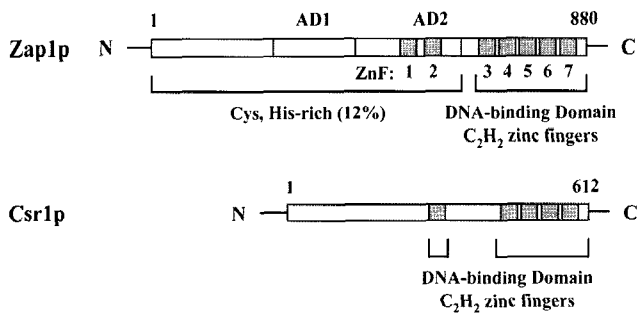

Fig. 1. Schematic comparisons of *C. albicans* Csr1 and *S. cerevisiae* Zap1 proteins.
Shown are the transcriptional activation domains (AD1 and AD2), the DNA-binding domain, and the N-terminal region of high Cys and His (12%) in Zap1. Boxes labeled with 1–7 indicate the C_2H_2-type zinc fingers found in Zap1 and Csr1. The amino acid sequence of Csr1 shows 34% identity and 49% positivity with that of Zap1.

csr1/csr1 Deletion Mutants Show a Severe Growth Defect Under Zinc-limiting Condition

In *S. cerevisiae*, zinc uptake is mediated by the Zrt1 and Zrt2 zinc transporters found in the plasma membrane [17, 27]. Mobilization of zinc stored in the vacuole is mediated by the Zrt3 transporter [5]. The expressions of *ZRT1*, *ZRT2*, and *ZRT3* increase in zinc-limited cells and are shut off at high zinc levels [7, 17]. The Zap1 transcriptional activator

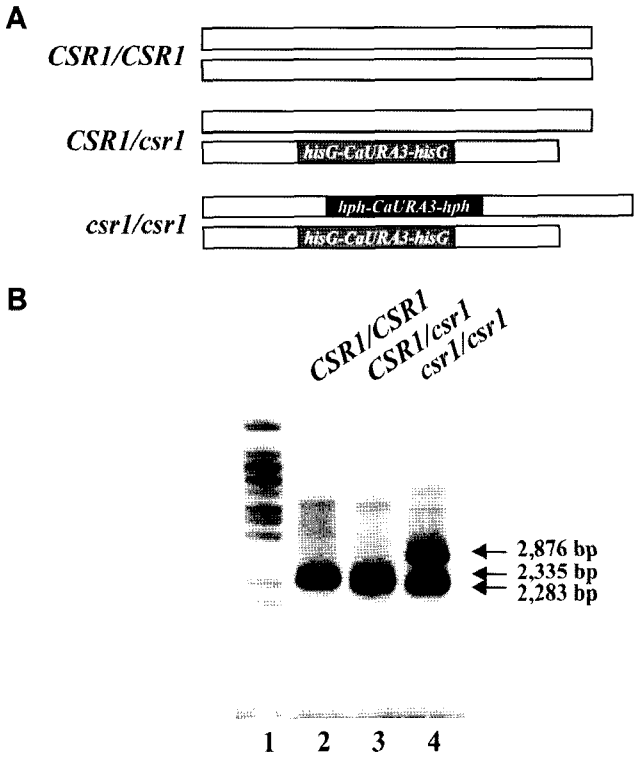

Fig. 2. Disruptions of *CSR1*.
A. Strategy for the sequential disruptions of two copies of *CSR1*. Internal fragments of the *CSR1* open reading frame were replaced by *hisG-CaURA3-hisG* or *hph-CaURA3-hph* cassettes (see Materials and Methods for the constructions of the disruption plasmids). **B.** Confirmation of *CSR1* disruption by PCR analysis. The *CSR1*, *csr1:: hisG-CaURA3-hisG*, and *csr1:: hph-CaURA3-hph* alleles are amplified as 2,335 bp, 2,283 bp, and 2,876 bp fragments, respectively. The band in lane 3 is a doublet of 2,335 bp and 2,283 bp.

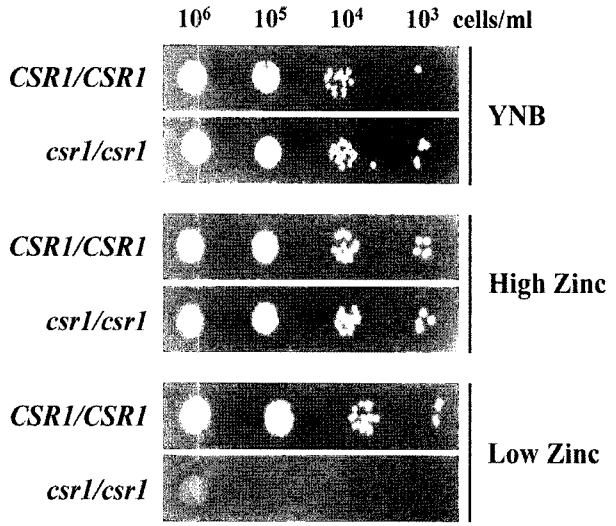

Fig. 3. Growth defects of the *csr1/csr1* mutant strain on zinc-limited media.
Overnight-grown cells of wild-type (CAI4) and *csr1/csr1* mutant (JKC53) strains were serially diluted, spotted on YNB, high-zinc (1 mM $ZnCl_2$) media, and low-zinc (100 μM $ZnCl_2$) media and grown at 30°C for 3 days.

is directly responsible for this regulation. DNA microarray analysis suggested that Zap1 controls the expression of as many as 42 genes in response to zinc status [17].

It was previously reported that the *zap1* mutant in *S. cerevisiae* was unable to grow under zinc-limiting conditions [3]. In order to address the question whether Csr1 is also involved in the zinc homeostasis in *C. albicans*, we constructed a *csr1/csr1* mutant strain by deleting sequentially two copies of *CSR1* (see Materials and Methods, Figs. 2A and 2B). Under zinc-limited growth conditions, the *csr1/csr1* mutants showed a severe defect in growth compared with a wild-type strain (Fig. 3). These results suggest that *CSR1* is essential for the zinc homeostasis in *C. albicans*.

csr1/csr1 Mutants Show Defects in Filamentous Growth

To address whether the perturbation of zinc homeostasis caused by deletion of *CSR1* leads to any phenotypes in filamentous growth, we compared the growth morphology of *csr1/csr1* mutants on various hyphae-inducing media. Cells were plated on serum-containing YEPD media and incubated at 37°C for 2–7 days (Fig. 4A). The *csr1/csr1* mutants were defective in hyphal growth as compared with a wild-type strain. These defects were recovered by reintroduction of a wild-type *CSR1* gene on a plasmid.

In liquid media at 37°C, germ-tube formation (the initial stage of hyphae formation) occurs rapidly within the first few hours of incubation. We observed that wild-type cells formed abundant germ tubes in all three inducing media, 10% serum, or Lee's and Spider media. However, germ-tube formation of the *csr1/csr1* mutant was reduced as compared with a wild-type strain (Fig. 4B).

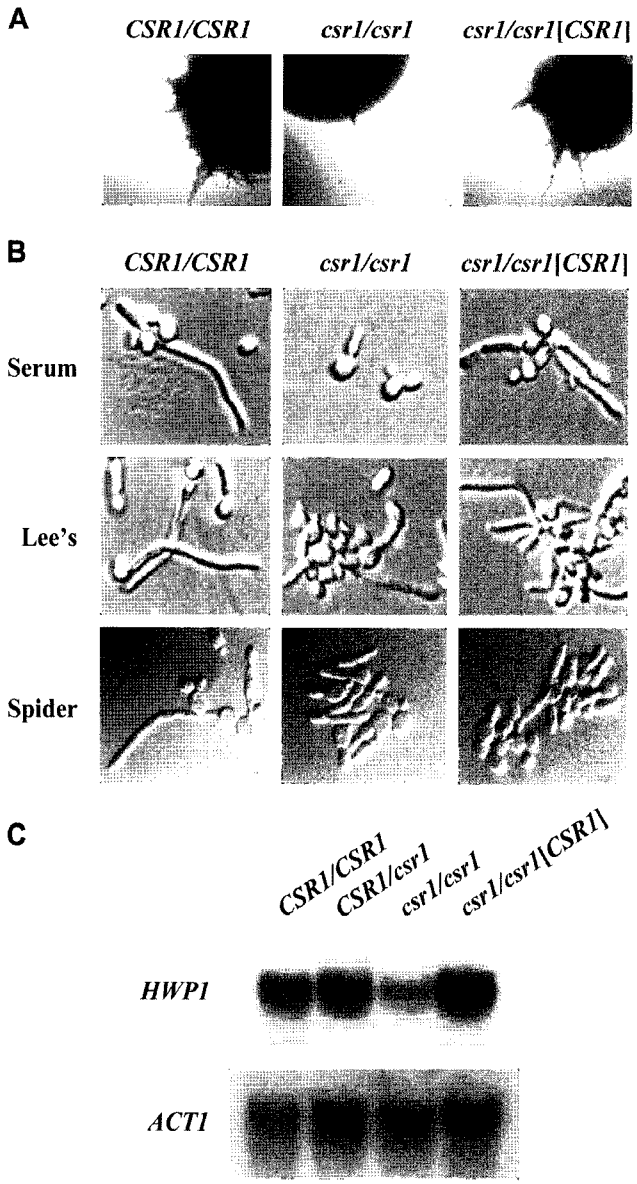

Fig. 4. Filamentation defects of the *csr1/csr1* mutant strain.
A. Colony morphologies of wild-type (SC5314), *csr1/csr1* (JKC53), and *csr1/csr1* [*CSR1*] mutants grown on solid serum-containing media at 37°C are shown. Colonies were photographed at the same magnification (4×). **B.** Cell morphologies of wild-type (SC5314), *csr1/csr1*, and *csr1/csr1* [CSR1] strains grown in liquid serum-containing, liquid Lee's, and liquid Spider media are shown. Cells were grown in YEPD media at 30°C and then shifted to the indicated inducing medium. Cells were photographed at the same magnification (40×). **C.** Northern blot of *HWP1* transcripts from wild-type (SC5314), *csr1/csr1*, and *csr1/csr1* [CSR1] strains. *ACT1* was used as an internal control.

To examine whether the filamentation defect of *csr1/csr1* is associated with the expression of the hyphae-specific genes, we analyzed the level of *HWP1* transcripts in a wild type and the *csr1/csr1* mutant strain. The *HWP1* gene encodes a glycosylphosphatidylinositol-modified cell wall protein and its expression is induced during the filamentous growth [15]. Nothern blot analysis shows that the *HWP1* transcript level was significantly reduced in the

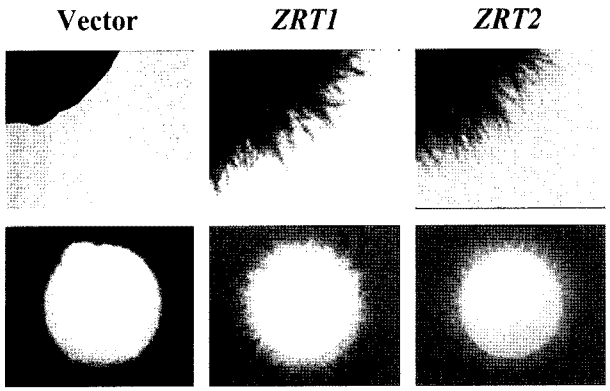

Fig. 5. Suppression of the *csr1/csr1* mutant strain by *ZRT1* and *ZRT2* overexpression.
Hyphal growth of the mutant strain JKC53 (*csr1/csr1*) containing vector pRC18, *ZRT1* plasmid (pRC18-ZRT1), or *ZRT2* plasmid (pRC18-ZRT2) was tested on Spider media. After 4 days of incubation, the colonies were photographed. Upper panels are the enlarged photos of the same colonies shown in the lower panel.

csr1/csr1 mutant (Fig. 4C). These results are in good correlation with the filamentation defects of the *csr1/csr1* strain.

C. albicans Homolog of Zinc Transporter Zrt1 or Zrt2 Suppresses the Filamentation Defect of *csr1/csr1*

The filamentation defect of the *csr1/csr1* mutation could have resulted from the failure of inducing the transcription of zinc transporter genes in *C. albicans*. Zinc uptake in *S. cerevisiae* is mediated primarily by two different systems in the plasma membrane: a high affinity system encoded by *ZRT1*, and a lower affinity system encoded by *ZRT2* [27]. To study the functional relationship of the transcription factor Csr1 with zinc transporters in *C. albicans*, we identified the *C. albicans* ORFs of *ZRT1* and *ZRT2* by using BLAST search. CaZrt1 showed an identity of 26% and a positivity of 47% when compared with ScZrt1. CaZrt2 showed an identity of 48% and a positivity of 65% when compared with ScZrt2 (amino acid alignment, data not shown).

High copy-number plasmid carrying *ZRT1* or *ZRT2* was introduced into the *csr1/csr1* mutant strain and the filamentation phenotypes were analyzed. As shown in Fig. 5, overexpression of *C. albicans ZRT1* or *ZRT2* suppressed the hyphal growth defect of the *csr1/csr1* mutant strain on Spider medium. From these results, we suggest that the filamentation phenotype of *C. albicans* is closely associated with the zinc level in the cells and that the *CSR1* gene plays a critical role in this regulation.

Discussion

Our data indicate that Csr1 is likely to be a functional homolog of the zinc-responsive transcription factor Zap1. Amino acid sequence analysis showed a high similarity between Csr1 and Zap1, mostly at the C-terminal zinc-finger region. Both the growth phenotypes of the *csr1/csr1* mutant strain under zinc-limited growth conditions and the suppression analysis of the *csr1/csr1* mutant with *ZRT1* or *ZRT2* overexpression clearly demonstrate the important role of *CSR1* in the zinc-responsive regulation. In *S. cerevisiae*, Zap1 is a transcription factor and plays a major role in inducing the expression of several genes under zinc-limited growth conditions. The Zap1 protein binds to a conserved sequence, called the zinc-responsive elements (ZRE), present in the promoters of *ZRT1*, *ZRT2*, *ZRT3*, and *ZAP1*. Target genes regulated by Zap1 include these *ZRT1*, *ZRT2*, and *ZRT3* zinc transporter genes and the *ZAP1* gene itself. In *C. albicans*, our study suggests that Csr1 functions upstream of Zrt1 and Zrt2, possibly as a zinc-responsive transcription factor.

Previously, a limited number of reports have addressed the importance of zinc ion in the hyphal development and pathogenicity of *C. albicans*. These studies are mainly at the physiological aspect. The micromolar concentrations of zinc ion suppress the mycelial formation *in vitro* [2]. In addition, a depression of the *in vivo* level of zinc has been implicated in the pathogenesis of *C. albicans* in individuals suffering from a disease related to zinc deficiency [5]. Mild zinc deficiency is associated with recurrent vaginal candidiasis and may play a role in the susceptibility of women to recurrent vaginal candidiasis [6]. In the present report, we introduced deletion mutations of *CSR1* in a *C. albicans* strain, which appeared to perturb the intracellular levels of zinc ion. The *csr1/csr1* mutant strain showed severe growth defect in a zinc-limited growth condition. The association of the zinc-related growth defect and the filamentation defect of the *csr1/csr1* mutant clearly supports the idea that zinc is a critical factor for the pathogenicity of *C. albicans*.

We identified *C. albicans ZRT1* and *ZRT2*, genes for the putative zinc transporters at the plasma membrane. Our preliminary data indicate that their expressions are regulated during the yeast-to-hyphal transition and dependent on Csr1 (data not shown). Further characterization of Zrt1 and Zrt2 in relation to the transcription factor Csr1 would unveil more about the zinc-responsive processes in *C. albicans*.

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