International Microbiology
https://doi.org/10.1007/s10123-022-00264-7

ORIGINAL ARTICLE

# Tec1 and Ste12 transcription factors play a role in adaptation to low pH stress and biofilm formation in the human opportunistic fungal pathogen *Candida glabrata*

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Received: 28 April 2022 / Revised: 1 July 2022 / Accepted: 4 July 2022
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**Abstract**
Eukaryotic cells respond to environmental cues through mitogen activated protein kinase (MAPK) signaling pathways. Each MAPK cascade is specific to particular stimuli and mediates specialized responses through activation of transcription factors. In the budding yeast, *Saccharomyces cerevisiae*, the pheromone-induced mating pathway and the starvation-responsive invasive growth/filamentation pathway generate their distinct outputs through the transcription factors Ste12 and Tec1, respectively. In this study, we report the functional characterization of these transcription factors in the closely related human opportunistic pathogenic yeast *Candida glabrata*. Two homologues each for *S. cerevisiae TEC1* and *STE12* were identified in *C. glabrata*. Both *C. glabrata* Tec1 proteins contain the N-terminal TEA DNA-binding domain characteristic of the TEA/ATTS transcription factor family. Similarly, the DNA-binding homeodomain shared by members of the highly conserved fungal Ste12 transcription factor family is present in N-terminus of both *C. glabrata* Ste12 transcription factors. We show that both *C. glabrata STE12* genes are at least partial functional orthologues of *S. cerevisiae STE12* as they can rescue the mating defect of haploid *S. cerevisiae ste12* null mutant. Knockout of one of the *STE12* genes (ORF CAGL0H02145g) leads to decreased biofilm development; a stronger biofilm-impaired phenotype results from loss of both *CgSTE12* genes in the double deletion mutant (*Cgste12*ΔΔ). The transcript levels of one of the *TEC1* genes (ORF CAGL0M01716g) were found to be upregulated upon exposure to low pH; its deletion causes slightly increased sensitivity to higher concentrations of acetic acid. Heat shock leads to increase in mRNA levels of one of the *STE12* genes (ORF CAGL0M01254g). These findings suggest a role of Tec1 and Ste12 transcription factors in the regulation of some traits (biofilm formation, response to low pH stress and elevated temperature) that contribute to *C. glabrata*'s ability to colonize various host niches and to occasionally cause disease.

**Keywords** *Candida glabrata* · Transcription factors · Biofilm

Among the microorganisms that colonize the oral cavity and gastrointestinal tract of most healthy humans are the yeasts belonging to the genus *Candida* (Kaur et al. 2005). Like other commensal microorganisms, *Candida* species are controlled by the innate immune system and by other competing microbes (Roetzer et al. 2011). However, in immunocompromised persons (e.g. diabetic, cancer and organ-transplant patients), elderly persons, long-term hospitalized patients with intravenous catheters and persons receiving antibiotic treatment, these fungi behave as opportunistic pathogens and can cause a range of diseases from superficial mucosal infections to systemic blood stream infections (Brunke and Hube 2013). *C. albicans* is the most frequent cause of candidiasis, followed in many regions of the world by *C. glabrata*. The incidence and prevalence of infections by *C. glabrata* is increasing (Kumar et al. 2019). *C. glabrata* is a haploid, budding yeast that exhibits high tolerance to commonly used azole antifungal drugs and has been shown to

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Published online: 12 July 2022

acquire resistance to recently developed echinocandins that inhibit cell wall synthesis (Katiyar et al. 2012). *C. glabrata* infections are associated with high morbidity and mortality, making it a fungal pathogen of high clinical significance and relevance.

*C. glabrata* is much more closely related to the budding yeast *Saccharomyces cerevisiae* than to *C. albicans* and other *Candida* species of the CTG clade. However, like *C. albicans*, *C. glabrata* survives and replicates in macrophages, evades immune response, has efficient stress (heat, pH, osmotic, oxidative, starvation) response mechanisms and shows colony switching. Pathogenicity seems to have evolved independently in *C. albicans* and *C. glabrata*; the two species have some shared and a few unique virulence attributes. Both species express large families of cell wall proteins that allow adherence to host cells and biofilm formation (Groot et al. 2013). Aspartyl proteases (secreted in case of *C. albicans* and GPI-linked in case of *C. glabrata*) are key virulence factors for both fungi (Naglik et al. 2003; Kaur et al. 2007). In contrast to *C. albicans*, *C. glabrata* does not form hyphae. A unique feature of *C. glabrata* are the *EPA* (epithelial adhesin) genes that are regulated by a sub-telomeric silencing machinery and play a key role in cell adhesion and biofilm formation (Las et al. 2003; Cormack et al. 1999; Iraqui et al. 2005). Like in other successful opportunistic pathogens, several signal transduction pathways and transcriptional regulatory networks have evolved in *C. glabrata*, which enable it to respond to changes and challenges offered by the host environment. Elucidating the roles of transcription factors that are the targets of these signaling pathways will aid in understanding *C. glabrata* commensal and infection strategies. The aim of this study is to investigate the regulation of *C. glabrata* virulence traits by two such transcription factors, Tec1 and Ste12.

In *Saccharomyces cerevisiae*, adhesion and biofilm formation are mediated by the GPI-anchored cell wall protein FLO11, whose expression is regulated by the transcription factors Tec1 and Ste12 (Andersen et al. 2014). In *C. albicans*, Tec1 regulates hyphal formation which is required for macrophage evasion. *C. albicans* Tec1 also regulates serum-induced expression of type 4, 5 and 6 isogenes of secreted aspartyl proteases and is required for virulence in a murine model of systemic candidiasis (Schweizer et al. 2000). *S. cerevisiae* Tec1 (Ty1 enhancement control), the founding member of the TEA/ATTS transcription factor family, was initially identified as a regulator of Ty1 retrotransposon expression (Laloux et al. 1990). The TEA (human TEF-1, *S. cerevisiae* Tec1 and *Aspergillus nidulans* AbaA) or ATTS (*A. nidulans* AbaA, human TEF-1, *S. cerevisiae* Tec1 and *Drosophila melanogaster* Scalloped) transcription factors regulate cellular differentiation and morphogenesis in eukaryotes. They are characterized by an N-terminal DNA-binding domain (TEA domain) that recognizes and binds TEA consensus sequence (TCS) elements (5′-CATTCC-3′ and 5′-CATTCT-3′) in target promoters (Andrianopoulos and Timberlake 1994). The Ste12-like transcription factor family is exclusive to fungi where it regulates development and pathogenicity (Hoi and Dumas 2010). One of the two *C. glabrata STE12* genes has been shown to be required for virulence (Calcagno et al. 2003). *S. cerevisiae* Ste12 (Sterile) was originally identified from a sterile mutant and regulates pheromone-responsive gene expression in haploid cells during mating (Errede and Ammerer 1989). *S. cerevisiae* Tec1 and Ste12 can interact and are required for haploid invasive growth in response to glucose depletion and for nitrogen starvation induced pseudohyphal growth in diploid cells. Ste12 is the target of the invasive growth/filamentous growth mitogen-activated protein kinase (MAPK) Kss1 and of the pheromone-responsive mating pathway MAPK Fus3 (Cullen and Sprague 2012). The *C. albicans* homologue of Ste12, Cph1, regulates virulence in association with the Efg1 transcription factor (Lo et al. 1997). Ste12-like transcription factors are important regulators of pathogenicity in both animal pathogens (*Cryptococcus neoformans*) and plant pathogenic fungi (*Fusarium oxysporum*, *Magnaporthe grisea*) (Hoi and Dumas 2010).

In this study, we have generated deletions of *TEC1* and *STE12* genes in *C. glabrata* and performed phenotypic analysis of *Cgtec1*Δ and *Cgste12*Δ null mutants. Our observations suggest that Tec1 and Ste12 transcription factors are involved in response and tolerance to low pH and in biofilm development respectively in *C. glabrata*.

## Materials and methods

### Strains and culture conditions

The wild-type *C. glabrata* strain used in this study is ATCC 2001 (CBS138) (ATCC collection). The deletions of *CgTEC1* and *CgSTE12* genes were generated in this strain background (Table 1). *C. glabrata* strains were routinely maintained on YPD (1% yeast extract, 2% peptone, 2% glucose) agar. Synthetic complete (2% glucose, 0.67% yeast nitrogen base containing ammonium sulphate, 1 × amino acids) medium was set to pH 5.5 and 2 using 0.1 M citrate buffer and HCl/NaOH. Logarithmic phase cultures were obtained by inoculating overnight cultures in fresh medium followed by incubation at 30 ℃ with shaking at 200 rpm for 4 h.

### Complementation of *S. cerevisiae ste12*Δ strain

Haploid *S. cerevisiae ste12*Δ strain was generated by a gene deletion strategy similar to that described for *Cgtec1*Δ and *Cgste12*Δ strains. *STE12* ORF was replaced by *LEU2* gene

**Table 1** *Candida glabrata* strains used in this study

| *Candida glabrata* strain | Genotype | Reference |
|---|---|---|
| ATCC 2001 (CBS138) | Wild type | ATCC collection |
| *Cgtec1(1)*Δ | *Cgtec1(1)*Δ*::hph* | This study |
| *Cgtec1(2)*Δ | *Cgtec1(2)*Δ*::nat1* | This study |
| *Cgste12(1)*Δ | *Cgste12(1)*Δ*::nat1* | This study |
| *Cgste12(2)*Δ | *Cgste12(2)*Δ*::nat1* | This study |
| *Cgtec1*ΔΔ | *Cgtec1(1)*Δ*::hph Cgtec1(2)*Δ*::nat1* | This study |
| *Cgste12*ΔΔ | *Cgste12(1)*Δ*::hph Cgste12(2)*Δ*::nat1* | This study |

in the haploid *S. cerevisiae* strain BY4741 (*MAT*a *ura3 his3 met15 leu2*) (Brachmann et al. 1998). *CgSTE12(1)* and *CgSTE12(2)* ORFs were cloned at SpeI/XhoI and EcoRI/SalI sites respectively of the yeast episomal plasmid p426 GPD (ATCC® 87,361™) (Mumberg et al. 1995). This allows constitutive expression of the *CgSTE12* genes under the strong glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter. The resultant plasmids termed GPD-CgSte12(1) and GPD-CgSte12(2), and p426-GPD vector, were transformed into *Scste12*Δ strain (*MAT*a *ste12::LEU2 ura3 his3 met15 leu2*). A representative transformant from each of the three uracil prototrophic strains generated namely (*MAT*a *Scste12*Δ p426 GPD), (*MAT*a *Scste12*Δ *CgSTE12(1)*) and (*MAT*a *Scste12*Δ *CgSTE12(2)*) was patch mated with the *MAT*α strain BY4742 (*MAT*α *ura3 his3 lys2 leu2*) (Brachmann et al. 1998) (on YPD agar for 48 h at 30 ℃). Then, appropriate dilutions of the patches were spotted on synthetic complete (SC) agar containing histidine and without uracil, lysine, methionine and leucine and grown at 30 ℃ for 48 h.

## Biofilm formation assay

The biofilm forming ability of *Cgtec1*Δ and *Cgste12*Δ strains was compared with that of wild type using the previously described 96-well (microtitre) plate model of biofilm formation (Iraqui et al. 2005). Stationary phase cultures were obtained by growth in synthetic complete (SC) medium for 24 h at 37 ℃ under shaking conditions (200 rpm). Cells were harvested, washed with water and resuspended in SC medium at an $OD_{600} = 1$. One hundred microlitres of cell suspension was added per well of 96-well flat bottom microtitre plates followed by incubation at 37 ℃ for 24 h. Then, loosely attached cells were removed by three washes with water and one wash with PBS. The metabolic activity of strongly attached cells (which is a measure of biofilm development) was determined by the XTT reduction assay. One hundred microlitres of XTT-menadione solution (0.5 mg/ml XTT to which menadione was added at a final concentration of 1 μM) was added to each well containing washed biofilms. Following incubation for 90 min at 37 ℃, colorimetric changes were measured at 492 nm using a microplate reader. Biofilm formation by wild type was considered 100%; biofilm formation by *Cgtec1*Δ and *Cgste12*Δ mutants was calculated and expressed as a percentage of the wild type.

## Expression analysis of *CgTEC1* and *CgSTE12* genes by qRT-PCR

To determine the effect of low pH stress on expression of *CgTEC1* and *CgSTE12* genes, logarithmic phase wild-type cells grown in synthetic complete (SC, pH 5.5) medium at 30 ℃ were harvested, washed and then grown in synthetic complete (SC, pH 2) medium at 30 ℃ for 1 h (Bairwa and Kaur 2011). To determine the effect of elevated temperature on expression of *CgTEC1* and *CgSTE12* genes, logarithmic phase wild-type cells grown in synthetic complete (SC, pH 5.5) medium at 30℃ were harvested, washed and then grown in synthetic complete (SC, pH 5.5) medium at 42 ℃ for 2 h (Miyazaki et al. 2011). RNA was isolated using the acid phenol method. Total RNA was treated with DNase I (New England Biolabs). Removal of contaminating genomic DNA was confirmed by PCR using exon- and intron-specific primers for the *CgACT1* gene. First-strand cDNA synthesis was performed using ProtoScript II first-strand cDNA synthesis kit (New England Biolabs). The expression of *CgTEC1* and *CgSTE12* genes was quantified by SYBR Green I chemistry based real-time PCR analysis. Relative quantification was carried out using *CgACT1* as the reference. For each gene, fold change in expression upon exposure to low pH medium/heat stress relative to growth in optimum pH/temperature was calculated.

## Spot assays for determining susceptibility to acetic acid and heat stress

The sensitivity of *Cgtec1*Δ and *Cgste12*Δ deletion mutants to acetic acid and heat stress compared to the wild type was determined by previously described spot assays (Chen et al. 2012; Gregori et al. 2007). Overnight grown cultures in YPD were inoculated in fresh YPD medium at $OD_{600}$ of 0.1 and then grown at 30 ℃ with shaking at 200 rpm for 4 h to obtain logarithmic phase cultures ($OD_{600}$ of 1). These exponentially growing cultures were diluted 1:10,

1:100 and 1:1000. For acetic acid tolerance assays, 5 µl of each dilution was spotted on YPD agar (pH 4.5, adjusted with HCl) and on YPD agar (pH 4.5) supplemented with 60 mM acetic acid. The pH of the 8.7 M acetic acid stock solution used was adjusted to 4.5 using NaOH. Results were noted after incubating the plates at 30 ℃ for 48 h. For thermotolerance assays, 5 µl of each dilution was spotted on YPD agar plates. Plates were examined after incubation at 30 ℃ for 24 h and at 40 ℃ for 48 h.

## Results

### *Candida glabrata* has two TEA/ATTS transcription factors

Two TEA domain containing genes (CAGL0F04081g and CAGL0M01716g) were identified in *C. glabrata* using BLAST analysis. We have designated these genes *CgTEC1(1)* and *CgTEC1(2)*, respectively. In common with most *C. glabrata* genes, *CgTEC1(1)* and *CgTEC1(2)* do not contain introns; their open reading frames (ORFs) are 1848 bp and 1308 bp long, respectively. Like other TEA/ATTS transcription factor family members, both CgTec1 proteins contain an N-terminal DNA-binding TEA domain (66–76 amino acids). CgTec1(1) and CgTec1(2) have 25% and 22% identity respectively at the amino acid level to *S. cerevisiae* Tec1 (ScTec1). The TEA domains of both CgTec1 proteins have substantial sequence homology with TEA domains of other TEA/ATTS transcription factor family members (Fig. 1a).

### The two *C. glabrata STE12* genes encode members of the Ste12 family of fungal transcription factors

A search for orthologues of *S. cerevisiae STE12* in *C. glabrata* using BLAST analyses identified two genes: CAGL0M01254g and CAGL0H02145g. We have assigned them the names *CgSTE12(1)* and *CgSTE12(2)* respectively. *CgSTE12(1)* and *CgSTE12(2)* contain uninterrupted open reading frames (ORFs) of 1797 bp and 1887 bp, respectively. Both CgSte12 proteins contain at their N-terminus the DNA-binding homeodomain-like motif (Ste), which is present in all Ste12 family members. Predictably, both proteins lack the two C-terminal $C_2H_2$ zinc fingers that are found in the Ste12-like proteins of filamentous fungi but not in yeast Ste12. CgSte12(1) and CgSte12(2) have 32% and 28% amino acid sequence identity respectively to *S. cerevisiae* Ste12 (ScSte12). The homeodomain-like motifs (Ste domains) of both CgSte12 proteins have high sequence similarity to Ste domains of other fungal Ste12 transcription factors (Fig. 1b).

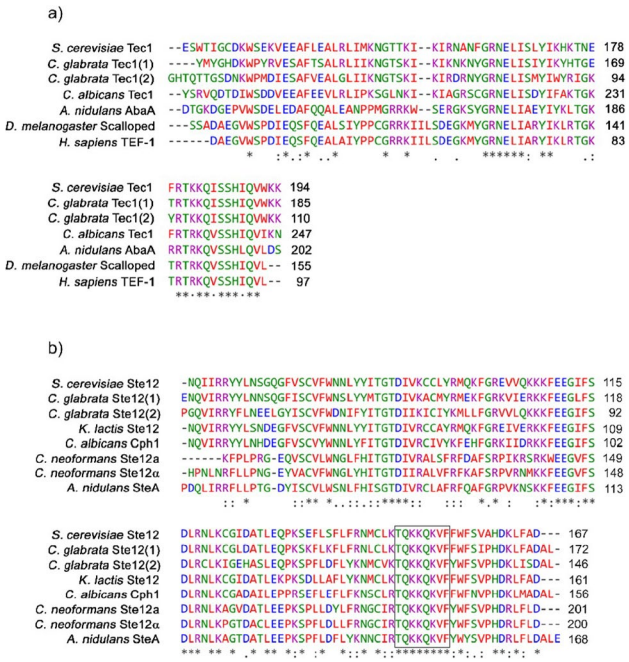


**Fig. 1** CgTec1 and CgSte12 proteins are members of the TEA/ATTS transcription factor family and fungal Ste12 transcription factor family, respectively. **a** Alignment of the N-terminal DNA-binding TEA domain of *Saccharomyces cerevisiae* Tec1 with orthologues from *Candida glabrata*, *Candida albicans*, *Aspergillus nidulans*, *Drosophila melanogaster* and *Homo sapiens* using Clustal Omega multiple sequence alignment tool (https://www.ebi.ac.uk/Tools/msa/clustalo/). The numbers at the sides of the sequences indicate the position of the amino acid (to the left of the number) in the protein sequence. The asterisk, colon and period symbols beneath the sequence represent that the amino acid residues at that position are identical, have strongly similar properties and have weakly similar properties, respectively. **b** Alignment of the N-terminally located homeodomain-like motif (Ste) of *Saccharomyces cerevisiae* Ste12 with Ste12 proteins from *Candida glabrata*, *Kluyveromyces lactis*, *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus nidulans*. The eight amino acid sequence "TQKKQKVF" (shown within a box) is part of a region that is highly conserved in all Ste12 proteins and has been shown to be crucial for DNA binding

### Generation of *Cgtec1*Δ and *Cgste12*Δ strains

*CgTEC1* and *CgSTE12* genes were deleted by homologous recombination-based strategy. *NAT1* (nourseothricin acetyltransferase) and *HPH* (hygromycin resistance) genes were used to replace *TEC1* and *STE12* ORFs. Linear deletion constructs for each gene consisting of 400–500 bp flanking regions and either of the two antibiotic selectable markers (*NAT1* or *HPH*) were generated by fusion PCR (Schwarzmüller et al. 2014) (Fig. 2a). The 5′ and 3′ regions flanking each ORF were amplified from wild-type genomic DNA; the *NAT1* resistance cassette (Sc*TEF1* promoter-*NAT1* ORF-Sc*CYC1* 3′ UTR) was amplified from the plasmid pBM16 (Ma et al. 2009) and the *HPH* resistance cassette (Sc*PGK1* promoter-*HPH* ORF-Sc*HIS3* 3′ UTR) was amplified from

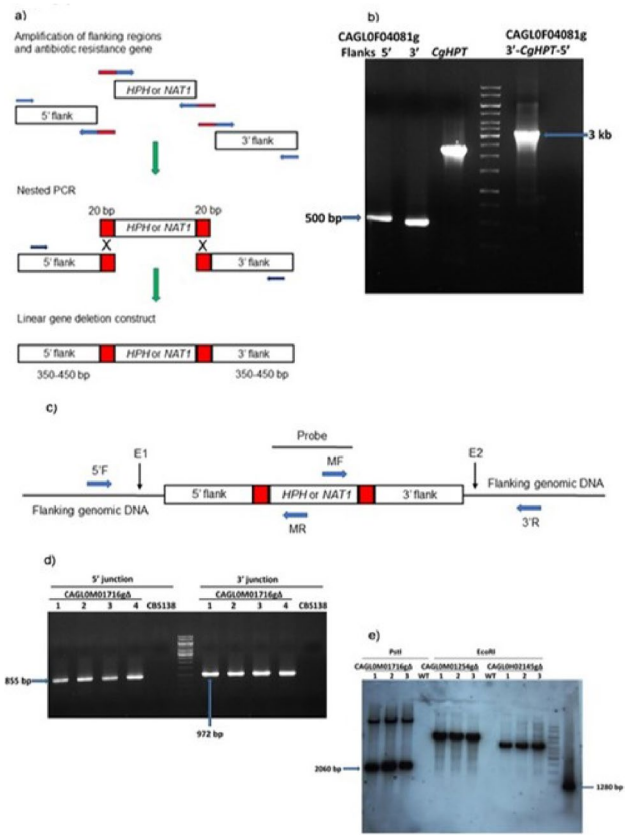


**Fig. 2** Deletion of *CgTEC1* and *CgSTE12* genes. **a** Schematic depiction of fusion PCR strategy for generating gene deletion cassettes (Brachmann et al. 1998). **b** Representative agarose gel showing deletion construct for *CgTEC1(1)*. **c** *CgTEC1*/*CgSTE12* ORFs were replaced by *NAT1* or *HPH* genes. *CgTEC1(1)* was deleted using the *HPH* gene while *NAT1* gene was employed to delete *CgTEC1(2)*, *CgSTE12(1)* and *CgSTE12(2)* genes. Homologous integration of linear deletion constructs at the target genomic locus was verified by PCR. Primers 5′F and 3′R lie external to the deletion cassette while primers MF and MR are on the antibiotic resistance gene (*NAT1*/*HPH*). E1 and E2 represent recognition sites for restriction enzymes used to digest genomic DNA for Southern analysis. Restriction endonucleases were chosen so that they have recognition sequences in the flanking genomic DNA on each side of the deletion cassette but not within the deletion construct. **d** Confirmation of gene deletion by PCR. Genomic DNA from putative transformants and wild type was used as template for PCR with primer pairs 5′F/MR and MF/3′R. PCR products of expected sizes were obtained for all deletion strains, and no PCR product was obtained when genomic DNA from wild type was amplified with these primers. Shown above is the result of these diagnostic PCRs for four independent transformants of *Cgtec1(2)*Δ. **e** Southern blot of *Cgtec1*Δ/*Cgste12*Δ strains and wild type. Genomic DNA of *Cgtec1*Δ/*Cgste12*Δ strains and wild type was digested with indicated restriction enzymes. For *Cgtec1(1)*Δ, 1.2 kb EcoRI fragment of *HPH* ORF was used as probe, whereas the entire 1.2 kb *NAT1* gene was used as probe for *Cgtec1(2)*Δ, *Cgste12(1)*Δ and *Cgste12(2)*Δ strains. Shown here is a blot for three independent transformants of *Cgtec1(2)*Δ, *Cgste12(1)*Δ and *Cgste12(2)*Δ strains. Bands of anticipated size were seen in each case and the use of selectable marker as probe meant that no fragment was labelled and visualized in the wild type

the plasmid pAP599 (Gujjula et al. 2016). The reverse primer for 5′ flank and forward primer for 3′ flank shared a 20-bp complementary region with the forward and reverse primers respectively for antibiotic resistance cassette amplification allowing the fusion of the three fragments to generate linear deletion cassettes (Table 2) (Schwarzmüller et al. 2014). The linear deletion cassettes were transformed into wild-type strain by a modified Lithium acetate/single-stranded carrier DNA/polyethylene glycol yeast transformation protocol (Srivastava et al. 2015). Gene deletion was confirmed in at least three independent transformants by PCR using gene-specific and marker-specific primers and by Southern hybridization using antibiotic resistance gene as probe (Fig. 2b–e). Double deletion strains were generated by deleting the other *CgTEC1*/*CgSTE12* gene in the respective single deletion background using either of the two available antibiotic selectable markers.

## Complementation of *CgSTE12 and CgTEC1* genes in *S. cerevisiae ste12*Δ and *tec1Δ* mutants

We deleted the *STE12* gene in the *S. cerevisiae MAT*a strain BY4741 and confirmed its sterile phenotype by patch-mating the *MAT*a *Scste12*Δ mutant with the *MAT*α strain BY4742 (Fig. 3a). It had been previously shown that expression of *CgSTE12(1)* led to rescue of mating defect in haploid *S. cerevisiae ste12*Δ mutant and of nitrogen-starvation filamentation defect in diploid *S. cerevisiae ste12*Δ mutant (Calcagno et al. 2003). Here we demonstrate that *CgSTE12(2)* also complements the mating defect of haploid *S. cerevisiae ste12*Δ strain (Fig. 3b). Regarding the invasive hyphae phenotype of CgTEC1, it was found that the phenotype was not restored in the *S. cerevisiae tec1*Δ strain (Fig. 4). Thus, the two *CgSTE12* genes may be functional homologues of *S. cerevisiae STE12* as they can restore one of the phenotypes resulting from the loss of *S. cerevisiae STE12*. In further experiments, to test the effect of cell wall and osmotic stress on the mutants, it was found that mutants had no effect on both (Fig. 5).

## Loss of both *CgSTE12* genes leads to reduced biofilm formation

Stationary phase *C. glabrata* cells have been shown to adhere to and form biofilm on plastic surfaces (Iraqui et al. 2005). One approach to measure biofilm development is to quantify strongly adhered cells using the colorimetric XTT reduction assay (Riera et al. 2012). Epa6 has been reported to be the principal adhesin required for biofilm formation in *C. glabrata* (Iraqui et al. 2005). We deleted the *EPA6* gene in the *C. glabrata* wild-type CBS138 strain using the *NAT1* (nourseothricin resistance) gene as selectable marker (*Cgepa6*Δ*::nat1*) and used the *epa6*Δ mutant as a control/

**Table 2** Primers used in this study

| | Primers used in this study: | |
|---|---|---|
| | Primer name | Sequence (5′-3′) |
| | For quantitative real-time PCR (qRT-PCR) | |
| | *CgACT1* F | GCGTTACCCAATCGAACACG |
| | *CgACT1* R | GTTCTTCTGGGGCGACTCTC |
| | *CgTEC1(1)* F | TAATGTAGCACCGCTGCCTG |
| | *CgTEC1(1)* R | GGCCACTTGTCGTGTCCATA |
| | *CgTEC1(2)* F | ACAGGCCAGCAGTGGTTTTA |
| | *CgTEC1(2)* R | GCCATTGTTGGGCGTCATTT |
| | *CgSTE12(1)* F | ACCTCCCAGCGTATAGTCCA |
| | *CgSTE12(1)* R | TGCGACTGTCGTCTTTGTGA |
| | *CgSTE12(2)* F | AACAAGATCCTGACCGAGGC |
| | *CgSTE12(2)* R | GATGGTCTCCTCGTTGGAGC |
| | *CgYPS1* F | CCACAAGCTCTAGTCACCCG |
| | *CgYPS1* R | AGTGTCATCGGACTGGCTTG |
| | *CgYPS4* F | GTTTGGCTTGGCTGCTTTCT |
| | *CgYPS4* R | TCTAGAGCCATTGGCAGCAA |
| | For gene deletion | |
| a) | Amplification of 5′ and 3′ flanking regions | |
| | (Overlap sequences for fusion PCR are in lowercase) | |
| | *ScSTE12* 5′ F | TGAAACAAACGCCGTTGTCC |
| | *ScSTE12* 5′ R | gcagggatgcggccgctgacTAGGTCTCGCTTGGGACACT |
| | *ScSTE12* 3′ F | cacggcgcgcctagcagcggAGAATTAAAAATGCGGGCCAGA |
| | *ScSTE12* 3′ R | ATTTTCCGATGGCCTTTGCC |
| | *CgTEC1(1)* 5′ F | TTTAAGCATTCCCTACCGCG |
| | *CgTEC1(1)* 5′ R | cacggcgcgcctagcagcggGGCGCAACGAAAGGAAATTG |
| | *CgTEC1(1)* 3′ F | gcagggatgcggccgctgacTGTCAATTTCACATCAGGGTTCA |
| | *CgTEC1(1)* 3′ R | CGCAAACATTTACAACTAGACCA |
| | *CgTEC1(2)* 5′ F | ACCCACATAAATCCGGTCCT |
| | *CgTEC1(2)* 5′ R | cacggcgcgcctagcagcggTTGAGGGCGTTATTTGTGGG |
| | *CgTEC1(2)* 3′ F | gcagggatgcggccgctgacCCCATCCGCCAACCAGATAT |
| | *CgTEC1(2)* 3′ R | TGGGTTTAAAATCGAACTTCCCT |
| | *CgSTE12(1)* 5′ F | ATCTAGACCCTCCTGACTCAG |
| | *CgSTE12(1)* 5′ R | cacggcgcgcctagcagcggGCGTGGAAATAGACTACCGG |
| | *CgSTE12(1)* 3′ F | gcagggatgcggccgctgacATCAGCCGCAAAAGATGGTG |
| | *CgSTE12(1)* 3′ R | TCCCAAGGTTCTGGTGTCAA |
| | *CgSTE12(2)* 5′ F | AGTTACCTGAAAACCAAAAGCCA |
| | *CgSTE12(2)* 5′ R | cacggcgcgcctagcagcggGGTCATCGCTTGCCATCATT |
| | *CgSTE12(2)* 3′ F | gcagggatgcggccgctgacAGACCACGCACCTTAAGACA |
| | *CgSTE12(2)* 3′ R | GTGGAAAATGGCTGCTGGAA |
| b) | Amplification of selectable markers | |
| | Amplification of Hygromycin resistance (*HPH*) cassette from plasmid pAP599 | |
| | *HPH* F | gtcagcggccgcatccctgcGCTCTAGAACTAGTGGATC |
| | *HPH* R | ccgctgctaggcgcgccgtgGACCATGATTACGCCAAGCTCG |
| | Amplification of nourseothricin resistance (*NAT1*) cassette from plasmid pBM16 | |
| | *NAT1* F | ccgctgctaggcgcgccgtgCATAGCTTCAAAATGTTTCTACTCC |
| | *NAT1* R | gtcagcggccgcatccctgcCCGCAAATTAAAGCCTTCGAG |
| | Amplification of *S. cerevisiae LEU2* gene | |
| | *ScLEU2* F | gtcagcggccgcatccctgcAAGTGCCACCTGACGTCTAA |
| | *ScLEU2* R | ccgctgctaggcgcgccgtgCGGGGTATCGTATGCTTCCT |

**Table 2** (continued)

| | Primers used in this study: | |
|---|---|---|
| | Primer name | Sequence (5′-3′) |
| c) | Nested primers | |
| | *Scste12*Δ N F | TTGAACAACTCTTCGCGGTC |
| | *Scste12*Δ N R | GCGATCATGTAGTTTTGGAGGT |
| | *Cgtec1(1)*Δ N F | AAAACAGGTTCTACAGGAACTG |
| | *Cgtec1(1)*Δ N R | GACGTCAAAATATAAGGTAAGCA |
| | *Cgtec1(2)*Δ N F | TTGCCAACGGTTCTCAAGAC |
| | *Cgtec1(2)*Δ N R | CACAGTGTTATTGCGATAAGTCA |
| | *Cgste12(1)*Δ N F | ACAAAATGGGATGGTAATGGAAC |
| | *Cgste12(1)*Δ N R | ACTAGAGAATGGTGATGACAAGC |
| | *Cgste12(2)*Δ N F | ACGGTTCAGTCATCTTGAGTTG |
| | *Cgste12(2)*Δ N R | TGAAAGAGAGCAACAACAAGCA |
| d) | Selectable marker and gene (5′ and 3′ flanking regions)-specific primers to confirm gene deletion | |
| | *HPH* 5′ R | CGAGCTTGGCGTAATCATGG |
| | *HPH* 3′ F | TGCAAACAAATCACGAGCGA |
| | *NAT1* 5′ R | TGTTTTGAAGTGGTACGGCG |
| | *NAT1* 3′ F | AAGGCTTTAATTTGCGGGCA |
| | *Cgtec1(1)*Δ 5′ F | AGATTGTGAATTTGCCCGACT |
| | *Cgtec1(1)*Δ 3′ R | TGGGGAAGTTTTGAATGGTTGA |
| | *Cgtec1(2)*Δ 5′ F | GCACTCTCTGACCATCTTGC |
| | *Cgtec1(2)*Δ 3′ R | GGTTCTTTGCACCGTTGGAT |
| | *Cgste12(1)*Δ 5′ F | ACAATGGACCACAACAACGG |
| | *Cgste12(1)*Δ 3′ R | GCTGAAGCGTTACAAAATGAGG |
| | *Cgste12(2)*Δ 5′ F | GCAGTATCGAGCTAGTGATTGT |
| | *Cgste12(2)*Δ 3′ R | AATGACTCCCTGCTCGTGAA |

reference strain in our biofilm assays. Consistent with original reports of *EPA6* deletion in BG2 strain, we observe that *epa6*Δ in CBS138 background too has a biofilm defective phenotype, with only 59.4% adherent cells compared with the wild type (Fig. 6). We observed that *Cgtec1(1)*Δ, *Cgtec1(2)*Δ, *Cgtec1*ΔΔ and *Cgste12(1)*Δ strains show wild-type levels of biofilm formation (Fig. 5). *Cgste12(2)*Δ is slightly affected with 80.2% adherent cells compared to the wild type while *Cgste12*ΔΔ mutant exhibited greater defect (65.1% adherent cells compared with the wild type) (Fig. 6).

## Low pH and heat shock lead to upregulation of *CgTEC1(2)* and *CgSTE12(1)*, respectively

*C. glabrata* colonizes host niches having large differences in pH and often experiences wide pH fluctuations in the same host environment over time. Consequently, it has evolved pH homeostasis mechanisms that allow it to maintain its intracellular pH even at very low extracellular pH (pH 2) (Bairwa and Kaur 2011). Adaptation to heat stress is another important strategy that enables *C. glabrata* to survive as an opportunistic pathogen. Hence, it was pertinent to investigate if the expression of *CgTEC1* and *CgSTE12* genes is affected in response to these relevant physiological stresses. It was observed that growth in low pH (pH 2) medium induces *CgTEC1(2)* (Fig. 7a) while exposure to high temperature (42 ℃) leads to increased expression of *CgSTE12(1)* (Fig. 7b). Members of the yapsin (Yps) family of glycosylphosphatidylinositol (GPI)-anchored aspartyl proteases have been shown to be upregulated under low pH conditions (Bairwa and Kaur 2011) and upon heat stress (Miyazaki et al. 2011). Thus, to validate our experiments, we also measured the mRNA levels of two *C. glabrata* yapsin genes namely *CgYPS1* and *CgYPS4*. We found expected increased transcript levels of *CgYPS4* and *CgYPS1* genes in response to low pH and high temperature stress, respectively (Fig. 7). We also found STE12(1) gene upregulation in response to heat shock even higher than that of the chosen as a positive control (YPS1).

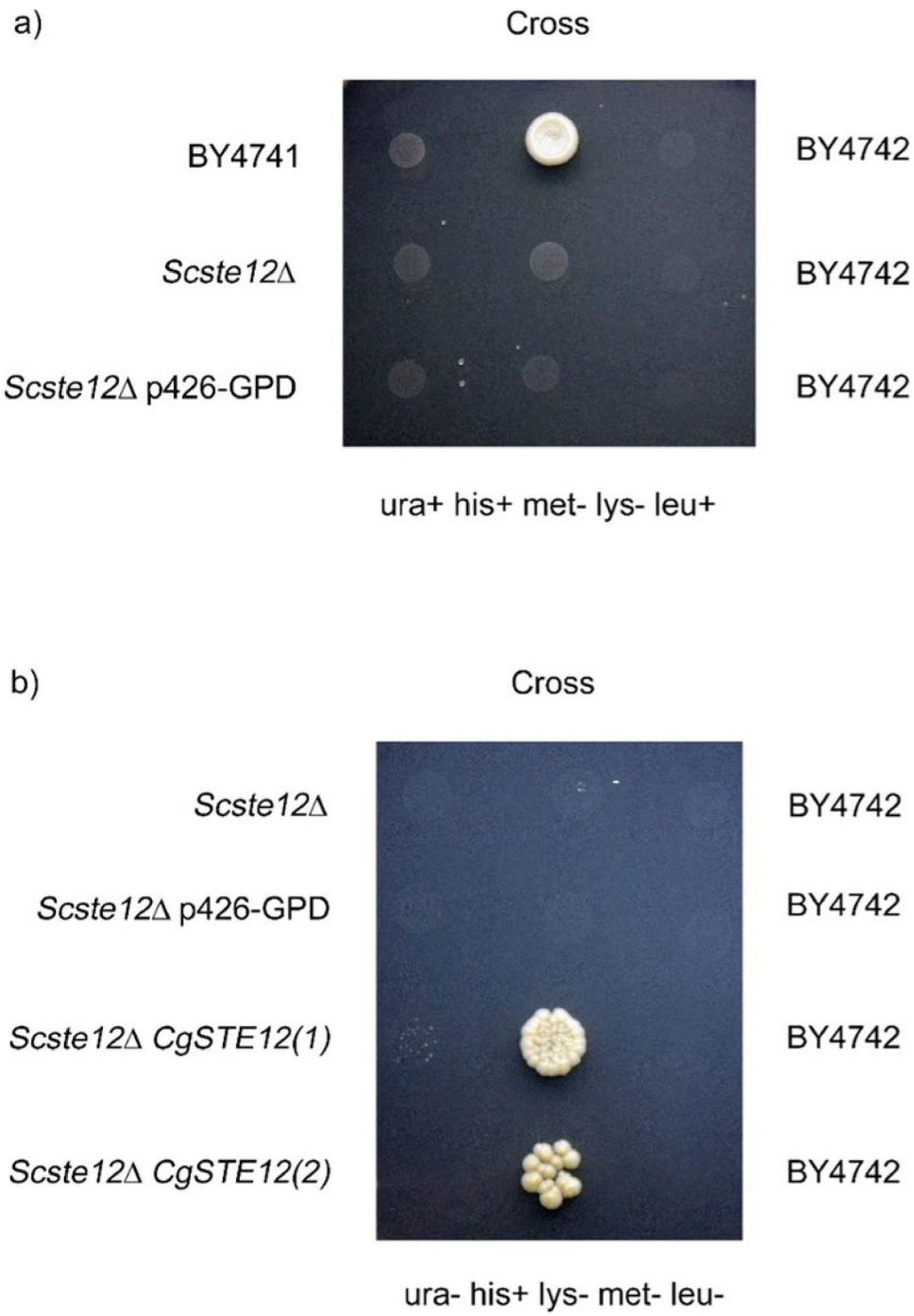


**Fig. 3** *CgSTE12* genes restore mating in *S. cerevisiae ste12*Δ strain. Indicated haploid *S. cerevisiae MAT*a strains were mated with haploid *MAT*α strain BY4742; suitable dilutions of the parental strains (left column for *MAT*a strains and right column for BY4742) and of the mating patches (centre column) were spotted on synthetic complete (SC) agar containing uracil, histidine, leucine but lacking methionine and lysine (**a**), and on SC agar containing histidine but lacking uracil, methionine, lysine and leucine (**b**), to confirm the sterile phenotype of *Scste12*Δ strain and to show that expression of either of the two *CgSTE12* genes is sufficient to provide mating ability to *Scste12*Δ mutant, respectively

## Deletion of *CgTEC1(2)* results in slightly increased susceptibility to acetic acid

Based on the upregulation of *CgTEC1(2)* in response to low pH, we decided to investigate if *CgTEC1(2)* is involved in tolerance to a weak organic acid (acetic acid). During colonization of the gastrointestinal and genitourinary tracts, *C. glabrata* cells encounter the presence of high concentrations of organic acids at low pH and thus have several mechanisms to cope with the adverse effects of this stress. We observe that the *Cgtec1(2)*Δ and *Cgtec1*ΔΔ null mutants showed slightly reduced growth on rich YPD medium (pH 4.5) supplemented with 60 mM acetic acid compared to the wild type, whereas the *Cgtec1(1)*Δ, *Cgste12(1)*Δ, *Cgste12(2)*Δ and *Cgste12*ΔΔ strains did not show any significant growth inhibition under the same conditions (Fig. 8). However, experiments using liquid media and higher acetic acid did not

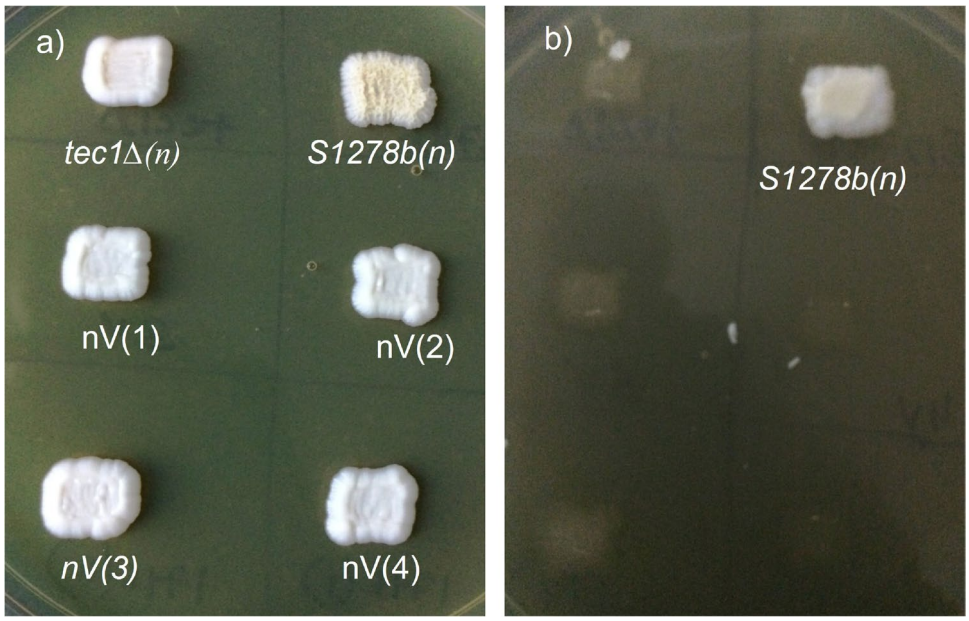


**Fig. 4** CgTEC1 gene does not restore the invasive hyphae phenotype in *S. cerevisiae tec1*Δ strain: *S. cerevisiae tec1*Δ strain was transformed with the cloned CgTEC1(2) in yeast episomal vector P426GPD and selected transformants on –Ura plate. For the invasive wash assay, four transformants were used. Cells patched on solid 2% YPD agar using inoculation loop, incubated for three days, flooded with water, shaken for 30 min and observed before (**a**) after the wash (**b**). Invasive wash assay shows all the four transformants (nV(1), nV(2), nV(3), nV(4)) that were obtained behaved like the mutant tec1Δ as they lacked the invasive hyphae, suggesting that CgTEC1(2) is not able to complement *S. cerevisiae tec1*Δ or the cloned gene is not getting expressed in *S. cerevisiae*

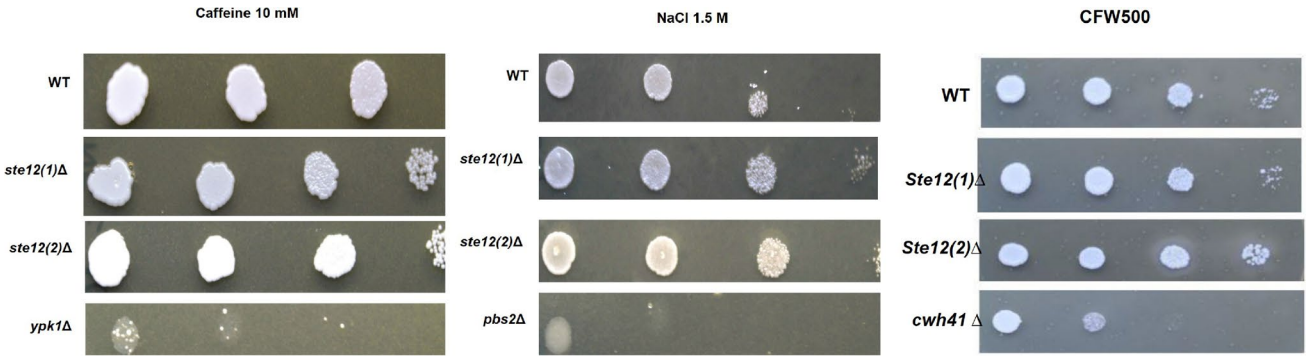


**Fig. 5** Cell wall and osmotic stress have no effect on CgSTE12 mutants. The growth of *Cgtec1*Δ and *Cgste12*Δ strains in the presence of cell wall (caffeine and CFW500) and osmotic stress (NaCl) agents was compared to that of the wild type by spot assays. Strains were grown to logarithmic phase in YPD medium. Five microlitres of serial dilutions (10-, 100- and 1000-fold) were spotted on YPD agar containing respective concentrations of stressors. Plates were observed after incubation at 30 ℃ for 48 h

show any significant difference in optical density of the grown cultures when compared to mutants (Suppl Fig. 1). Unlike the very high sensitivity to acetic acid reported for the *Cghaa1*Δ deletion mutant (Bernardo et al. 2017a), the *Cgtec1(2)*Δ and *Cgtec1*ΔΔ mutants show only slightly increased sensitivity to acetic acid compared to the wild type; the phenotype is weak/moderate. Since the antifungal activity of weak acids is due to their undissociated form (RCOOH), the pH of YPD agar and of the acetic acid stock solution were set at 4.5, close to the pKa of acetic acid (4.7).

## *CgSTE12* genes are not required for growth at elevated temperature

Taking note of the heat-induced expression of *CgSTE12(1)*, the growth of *Cgste12(1)*Δ mutant at high temperature (40 ℃) was compared to its growth at optimum temperature

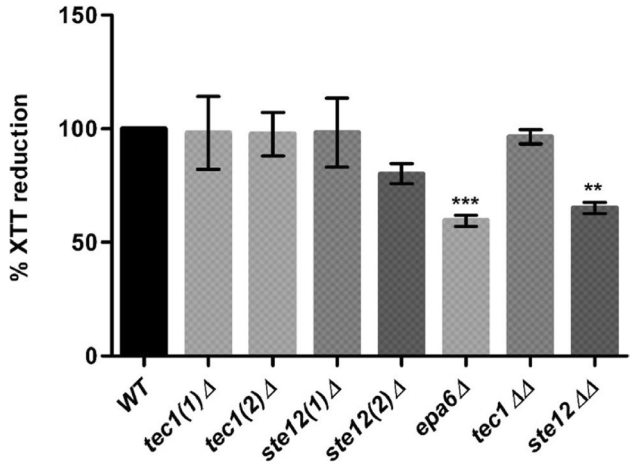


**Fig. 6** *CgSTE12* genes regulate biofilm development. Biofilm formation by *Cgtec1*Δ and *Cgste12*Δ mutants was monitored by the metabolic XTT reduction assay; the biofilm defective *epa6*Δ strain was used as a reference strain. The XTT reduced by the wild type (and hence biofilm formation) is considered 100%. XTT reduction by deletion mutants is expressed as percentage of the wild type. The results reported are the means ± standard error of means of three independent experiments. One-way ANOVA was used to test for statistically significant difference. ***$P < 0.0001$; **$P < 0.01$

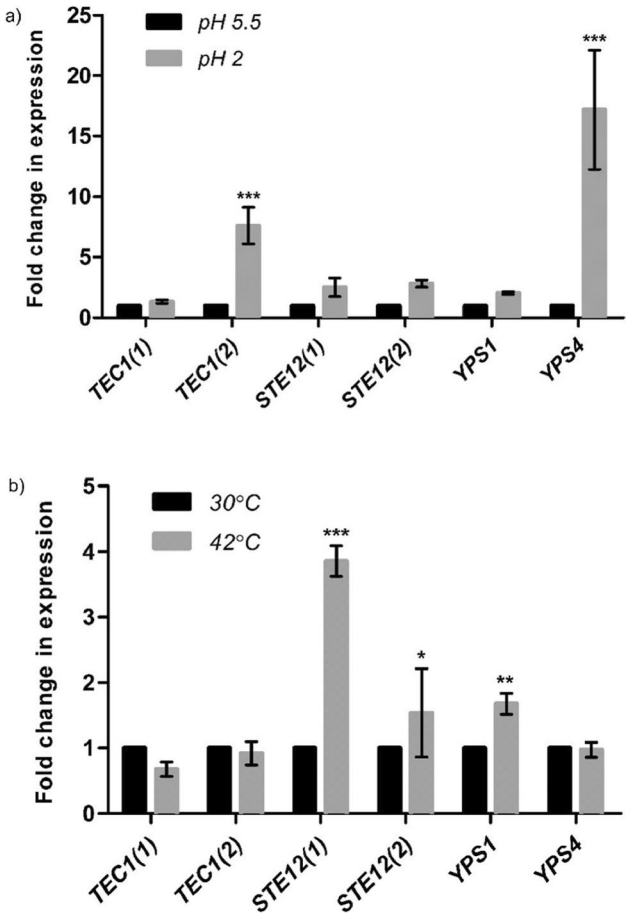


**Fig. 7** *CgTEC1(2)* and *CgSTE12(1)* are induced upon low pH and heat stress, respectively. **a** Determination of transcript levels of *CgTEC1* and *CgSTE12* genes in response to low pH stress by quantitative real time PCR (qRT-PCR). mRNA levels of *CgTEC1* and *CgSTE12* genes in wild-type logarithmic phase cells grown in synthetic complete (SC) pH 2 medium at 30 ℃ for 1 h (grey bars) were compared with mRNA levels of these genes in wild-type logarithmic phase cells grown in synthetic complete (SC) pH 5.5 medium at 30 ℃ for 1 h (considered to be 1, black bars). Transcript levels were normalized to transcript levels of the internal control *CgACT1*. Data represented was obtained from RNA extracted from three independent cell cultures, with each PCR reaction being set up in triplicates. **b** *CgTEC1* and *CgSTE12* gene expression upon heat shock was quantified by quantitative real-time PCR (qRT-PCR). Results are reported as fold change in expression after growth of logarithmic phase wild-type cells at 42 ℃ for 2 h (grey bars) relative to transcript levels after growth of logarithmic phase wild-type cells at 30℃ for 2 h (considered to be 1, black bars). The values shown are the means ± standard error of means after three independent experiments. Statistically significant differences (represented by asterisk) were determined by two-way ANOVA. ***$P < 0.001$; **$P < 0.01$; and *$P < 0.05$

(30 ℃) by spot assay (Chen et al. 2012). Like the wild-type strain, the *Cgste12(1)*Δ deletion strain showed no significant difference in growth at 30 ℃ and 40 ℃ (Fig. 9). Similar observations were obtained for the *CgTEC1* single and double deletion strains and for *Cgste12(2)*Δ and *Cgste12*ΔΔ strains. Thus, while *CgSTE12(1)* is upregulated in response to heat stress, CgTec1 and CgSte12 transcription factors do not appear to play a role in thermotolerance in *C. glabrata*.

## Discussion

During disease progression, fungal pathogens experience frequent and often highly harmful alterations in their environment. Consequently, adaptation programs and tolerance mechanisms to various stresses are essential features of fungal pathogenesis. Many of these survival strategies involve mitogen-activated protein kinase (MAPK) cascades. Although the MAPK pathways are highly conserved, the functions and roles of particular pathways in pathogenic fungi are sometimes different from homologous pathways in their closely related non-pathogenic counterparts. In the baker's yeast *S. cerevisiae*, the MAPKs Fus3, Kss1, Slt2 and Hog1 are involved in responses to pheromone, nutrient limitation, cell wall stress and hypertonic stress, respectively. This work aimed to establish the function of orthologues of the Kss1-responsive transcription factors, Tec1 and Ste12, in the human pathogenic yeast *C. glabrata*. In contrast to the single genes present in *S. cerevisiae* and *C. albicans*, *C. glabrata* has two *TEC1* and two *STE12* genes. Both *CgTEC1* and *CgSTE12* genes show a high degree of relatedness to *S. cerevisiae TEC1* and *STE12* at the sequence level. We report that both *CgSTE12* genes can complement a deletion of *STE12* in *S. cerevisiae* for the resulting mating defective/sterile phenotype, but

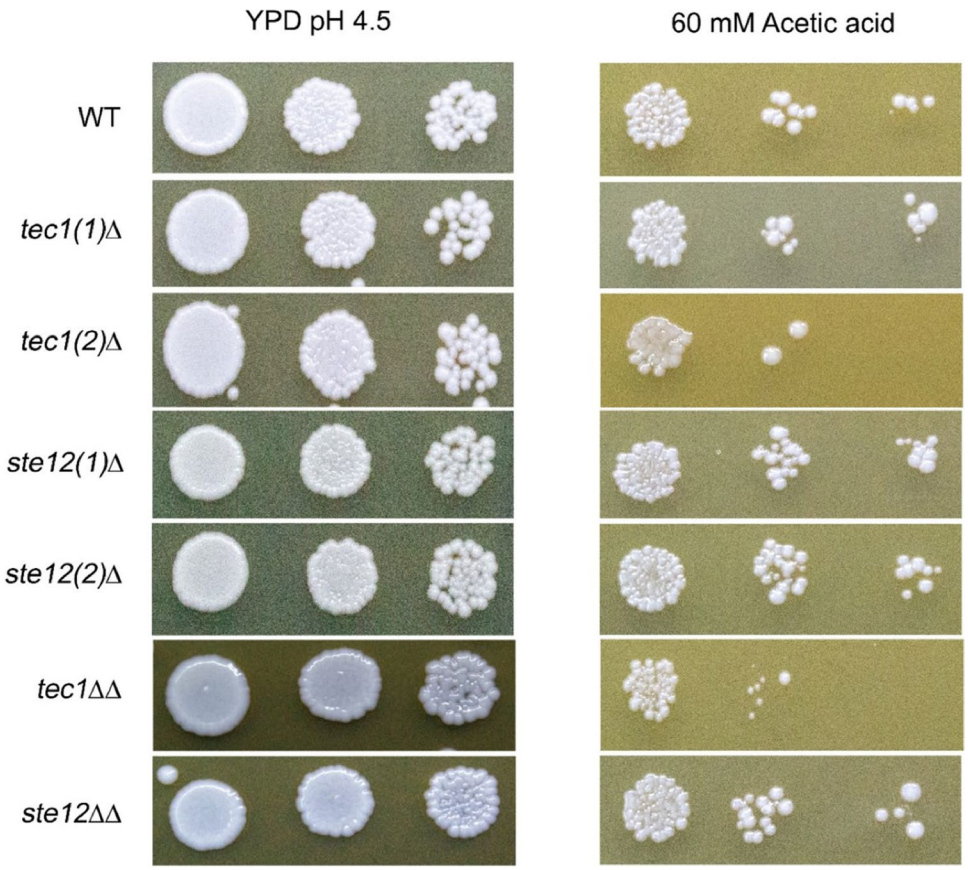


**Fig. 8** *Cgtec1(2)*Δ mutant displays slightly increased sensitivity to acetic acid. The growth of *Cgtec1*Δ and *Cgste12*Δ strains in the presence of acetic acid was compared to that of the wild type by spot assays (Srivastava et al. 2015). Strains were grown to logarithmic phase in YPD medium (pH 5.5). Five microlitres of serial dilutions (10-, 100- and 1000-fold) were spotted on YPD agar (pH 4.5, adjusted with HCl) and on YPD agar (pH 4.5) containing 60 mM acetic acid. The pH of the acetic acid stock solution used was set to 4.5 using NaOH. Plates were observed after incubation at 30 ℃ for 48 h

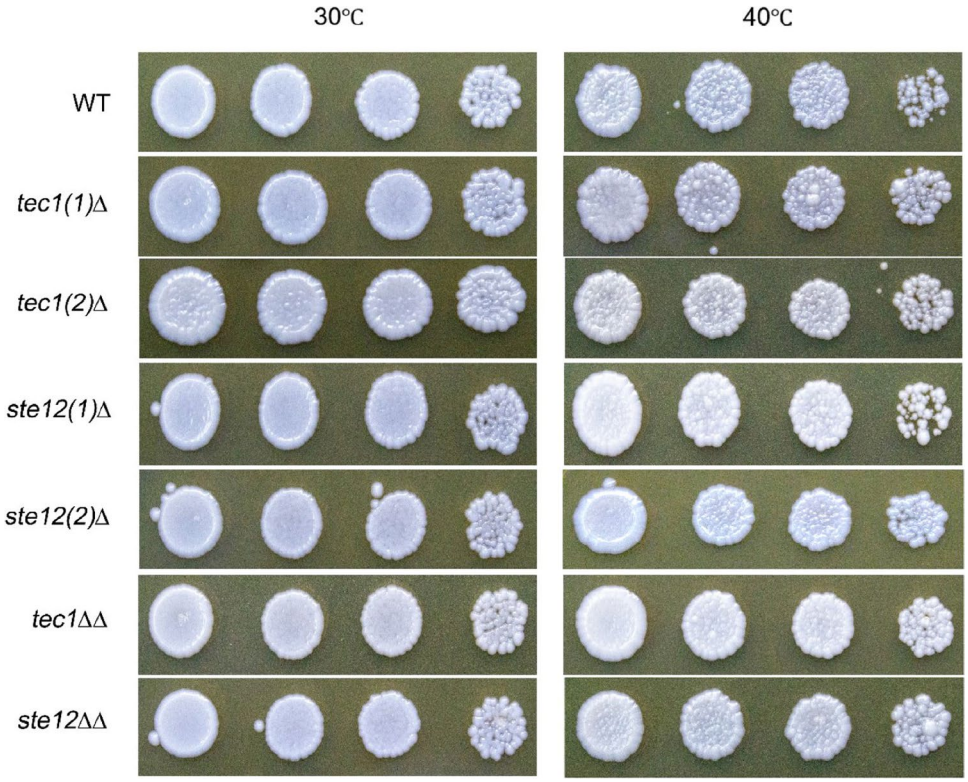


**Fig. 9** CgSte12 transcription factors are dispensable for growth at high temperature. Logarithmic phase cells were serially diluted (10-, 50-, 100- and 1000-fold), and 5 µl of each dilution was spotted on YPD agar. Plates were incubated at 30 ℃ for 24 h and at 40 ℃ for 48 h. Shown above is a representative result of three independent experiments

CgTEC1 gene was not able to complement TEC1 in S. cerevisiae. One of the *CgTEC1* genes, *CgTEC1(2)*, was induced in response to low pH; its deletion mutant was found to be slightly more sensitive to acetic acid. Knock-out of one of the *CgSTE12* genes, *CgSTE12(2)*, conferred a biofilm defective phenotype.

We would like to mention that a recent study (Cavalheiro et al., 2021) reported that *CgTEC1(2)* is required for biofilm formation in *C. glabrata* (Cavalheiro et al. 2021). In this investigation, *C. glabrata* biofilm formation on polystyrene was quantified by Presto Blue Cell Viability assay. We used the strategy of Iraqui et al. (2005) to determine *C. glabrata* biofilm formation on polystyrene using XTT reduction assay (Iraqui et al. 2005). Schwarzmuller et al. (2014) screened a *C. glabrata* deletion collection to identify biofilm mutants and reported no role for *CgTEC1(2)* in biofilm development (Schwarzmüller et al. 2014). Schwarzmuller et al. (2014) measured *C. glabrata* biofilm formation on polystyrene using fluorescein diacetate (FDA). We are at present unable to explain why our observations match with those of Schwarzmuller et al. (2014) but not with those of Cavalheiro et al. (2021).

Our observation that one of the *CgTEC1* genes has a role in response and tolerance to low pH, a role not seen for *S. cerevisiae TEC1*, may be explained by recent studies which show that while stress response pathways are highly similar in these two closely related yeasts, significant differences also occur. A common, immediate response to intracellular acidification is increased activity of plasma membrane $H^+$-ATPase (Pma1) and vacuolar $H^+$-ATPase to pump exceeding protons from the cytoplasm out of the cell or to the vacuole lumen, respectively. In both *S. cerevisiae* and *C. glabrata*, the transcription factor Haa1 has been shown to be the principal determinant of tolerance to acetic acid (Bernardo et al. 2017b). In response to high concentrations of acetic acid, CgHaa1 increases the expression of the CgPma1 proton pump and of multidrug resistance transporters of the major facilitator superfamily such as CgTpo3 that function to efflux acetate out of the cell (Bernardo et al. 2017b). The Sln1 branch of the HOG MAPK pathway also has a role in response to acetic acid stress in *S. cerevisiae* (Mollapour 2006). The Sln1 branch in *C. glabrata* ATCC 2001 strain is inactive because the MAPKKK *CgSSK2* in this strain lacks a functional kinase domain due to a point mutation (Gregori et al. 2007). This might explain why when compared to BG2 and other clinical isolates, ATCC 2001 is clearly more sensitive to acetic acid (Gregori et al. 2007). Alternatively, in response to acetic acid stress, CgTec1(2) may be activated by presently unknown pathway(s). Determining the acetic acid sensitivity of a *Cgtec1(2)*Δ mutant in the BG2 strain background might reveal whether loss of *CgTEC1(2)* causes sensitivity to acetic acid as a function of the strain background.

The calcineurin-Crz1 pathway has been shown to be essential for thermotolerance in *C. glabrata* (Chen et al. 2012). The calcineurin-Crz1 pathway regulates expression of cell wall remodeling proteins like CgYps1 in response to heat stress (Riera et al. 2012). In *S. cerevisiae*, the sterile vegetative growth (SVG) pathway comprises the MAPK-KKK Ste20, MAPKKK Ste11, MAPKK Ste7, MAPK Kss1 and transcription factor Ste12 functions to maintain cell wall integrity under hypotonic stress and heat shock in parallel with the protein kinase C-Mpk1/Slt2 MAPK pathway (Lee and Elion 1999). Our observation that while *CgSTE12(1)* transcript levels are induced at high temperature, the deletion of *CgTEC1* and *CgSTE12* genes does not affect growth at elevated temperature suggests a different role for this pathway in *C. glabrata*. Moreover, the expression of *CgSTE12(1)* even higher than *CgYps1* warrants further investigation.

Our preliminary findings indicate that the processes controlled by Tec1 and Ste12 transcription factors in *C. glabrata* may be different from those in the closely related *S. cerevisiae*. The challenges encountered by *C. glabrata* in its mammalian hosts are vastly different from those faced by *S. cerevisiae* in its ecological niches and thus many signaling pathways in these two close genetic relatives have diverged and have been rewired. Much further work needs to be performed to establish the regulatory networks of Tec1 and Ste12 in the clinically relevant opportunistic pathogenic yeast *Candida glabrata*.

## Conclusions

This study aimed to characterize two transcription factors (Tec1 and Ste12) in the opportunistic pathogenic yeast *Candida glabrata*. Our findings indicate a role of these transcription factors in low pH stress response and tolerance and biofilm formation which are among the many adaptations that allow *C. glabrata* to survive as a successful commensal and opportunistic pathogen.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s10123-022-00264-7.

**Acknowledgements** This article pays tribute to Late Professor Bharat Chattoo and Late Dr. J Manjrekar.

**Author contribution** Divya Purohit — conceptualized the work, performed experiments and contributed to manuscript writing.

Devarshi Gajjar — conceptualized the work and contributed to manuscript writing

**Funding** This research was funded by Department of Biotechnology, Ministry of Science and Technology, Government of India, grant number BT/PR8100/MED/29/707/2013. Divya Purohit is a recipient of a senior research fellowship from University Grants Commission, New Delhi, India.

**Data availability** Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

## Declarations

**Ethics approval and consent to participate** Not applicable.

**Consent for publication** Not applicable.

**Conflict of interest** The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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