Ras1 involved in the antifungal resistance of *Candida albicans* and the inhibition of farnesol on the resistance of biofilms

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Abstract

Background: Farnesol enhances the susceptibility of *Candida albicans* biofilms to antifungals, while the molecular mechanisms of this behavior are poorly understood. RAS1 regulates the hyphal growth of *C. albicans*, and farnesol inhibited hyphal growth by RAS1 regulation, while the role of RAS1 in the resistance of *C. albicans* biofilms and the molecular mechanism of the RAS1 in the farnesol-relevant antifungal capacity to *C. albicans* biofilms is still unknown. The study hypothesized that Ras1 involved in the antifungal resistance of *C. albicans* and the inhibition of farnesol on the resistance of biofilms.

Results: The susceptibility assays showed that RAS1 over-expressing strain (RAS1OE) increased the resistance of *C. albicans* in both planktonic and biofilm form to antifungals, while RAS1 deletion strain (*ras1* Δ/Δ) reduced that to antifungals. The SMIC50 of the antifungals were increased with the mature of the biofilms formed from the mutant and the wild strains. Exogenous farnesol decreased the resistance of RAS1OE to antifungals, including fluconazole, amphotericin B, itraconazole, caspofungin, terbinafine, 5-fluorocytosine and nystatin. The inhibitory effects of farnesol on the antifungal resistance of the biofilms from the RAS1OE were in accordance to almost all of the growth phases. Moreover, exogenous farnesol decreased the resistance of biofilms from RAS1OE more obviously than that from the wild strains ($P<0.05$). In addition, Morphological observation showed that that RAS1OE increased hyphal growth the biofilms, while *ras1* Δ/Δ reduced that of the biofilms. Compare to the wild-type strain, the inhibitory effects of farnesol on hyphal growth were more obvious to the RAS1OE, while less obvious to the *ras1* Δ/Δ .

Furthermore, farnesol reduced the level of Ras1 and the expression of RAS1 of the biofilms formed from the RAS1OE strain compared with those of the untreated controls at all studied phases. Moreover, farnesol reduced the level of Ras1 and the expression of RAS1 of the biofilms formed from RAS1OE more obviously than that from the wild strains.

Conclusions: Ras1 involved in the antifungal resistance of *Candida albicans*, and the inhibition of farnesol on the resistance of biofilm.

Background

The fungus *Candida albicans*, an opportunistic human pathogen, is commonly found in the biofilms of the oral cavity and gastrointestinal tract. The biofilms of *C. albicans* are significantly less susceptible to antimicrobial agents than that of the planktonic cells, which seriously weakens the effectiveness of antifungals [1]. Indeed, 65~80% of *C. albicans* infections were associated with biofilms [2, 3]. The extensive use of antifungals has led to the increased resistance of the biofilms to the few existing antifungals. Thus, it is crucial to explore the mechanisms of therapeutic or preventive strategies targeting biofilm-related infections.

Previous studies have demonstrated that farnesol prevented the germination of yeast cells into mycelia, repressing hyphal growth and biofilm formation [4, 5]. Further studies showed that farnesol enhanced the microbial susceptibility to antibiotics of *Staphylococcus aureus* [6], *Paracoccidioides brasiliensis* [7], *Streptococcus mutans* [8], and *Fusarium graminearum* [9]. Another *in vitro* study showed that farnesol inhibited the development of *C. albicans* biofilms formed from the resistant strains, and farnesol in combination with fluconazole, itraconazole and 5-fluorocytosine had synergistic effects against *C.*

albicans biofilms [10]. Farnesol possibly via regulation of the ergosterol biosynthesis pathway [11] to enhance the susceptibility of planktonic *C. dubliniensis* and *C. albicans* to fluconazole [6]. However, the exact mechanism underlying the farnesol-mediated increase in susceptibility to various antifungals is poorly understood in *C. albicans* biofilms.

C. albicans RAS proteins are small GTPases that can trigger the MAPK and cAMP signaling pathway, and involved in morphogenesis [4, 12], stress resistance [13] and the maintenance of the hyphal state [14]. Ras1 protein are highly conserved signaling proteins that play central roles in key physiological processes, in this process *RAS1* regulates a diverse array of phenotypes that are critical for both commensal and pathogenic lifestyles within the host [15]. Recent data suggest that MAPK and cAMP signaling pathways not only regulate the morphogenesis of *C. albicans* but also participate in the drug resistance of *C. albicans* biofilms [16]. However, the role of *RAS1* in the resistance of *C. albicans* biofilms was not discussed in these previous studies, which need to be further studied.

The molecular mechanism of the *RAS1* in regulating the farnesol-relevant antifungal capacity to *C. albicans* biofilms is still unknown. In the present study, we hypothesized that *RAS1* is associated to the antifungal resistance of *C. albicans* biofilm, and also involved in the regulation of farnesol on the resistance of *C. albicans* biofilm. To test this hypothesis, the antifungal resistance of *C. albicans* biofilms formed by *RAS1* mutant strains (including *RAS1* overexpressing strain (*RAS1OE*) and deletion strain (*ras1Δ/Δ*) and the wild strains was examined in the presence or absence of farnesol. Meanwhile, the morphological changes were examined using confocal laser scanning microscope (CLSM) and scanning electron microscopy (SEM). In addition, the effects of farnesol on the expression of *RAS1* and Ras1 in *C. albicans* biofilms formed from the studied strains were analyzed using RT-qPCR and western blot, respectively.

Results

Ras1 of the *C. albicans* biofilms is regulated by farnesol

The results showed that *RAS1* expression and Ras1 level were down-regulated in the biofilms of *C. albicans* SC5314 and the resistant strain exposed to farnesol at four different growth phases (6, 12, 24 and 36 h) ($P<0.01$) (Fig. 1).

C1: Untreated *C. albicans* SC5314; F1: farnesol-treated *C. albicans* SC5314; C2: untreated resistant strain; F2: farnesol-treated resistant strain. F1/C1 and F2/C2: Comparison of gene expression between the farnesol-treated and untreated groups of *C. albicans* biofilms.

RAS1 and Ras1 were down-regulated in the biofilms of *C. albicans* SC5314 and the resistant strain exposed to farnesol at four different growth phases. Farnesol: 200 μ M; *: $P<0.05$; **: $P<0.01$.

Ras1 involved in the resistance of *C. albicans* to antifungals

For the spot assay, *RAS10E* was more resistant to fluconazole (4 µg/ml) and itraconazole (0.5 µg/ml) than the wild-type strain (pCaEXP-CAI4) (Fig. 2). Moreover, *ras1Δ/Δ* was more susceptible to fluconazole (4 µg/ml), itraconazole (0.5 µg/ml), amphotericin B (2 µg/ml), nystatin (2 µg/ml), caspofungin (0.5 µg/ml), terbinafine (15 µg/ml) and 5-fluorocytosine than the wild-type strain (SN152) (Fig. 2).

In the spot assay, antifungals were added to the medium at a concentration of 4 µg/ml for fluconazole, 2 µg/ml for amphotericin B, 0.5 µg/ml for caspofungin, 15 µg/ml for terbinafine, 0.5 µg/ml for itraconazole, 2 µg/ml for nystatin and 8 µg/ml for 5-fluorocytosine. Then, 5µl of tenfold serial dilutions of the suspensions were spotted onto YPD plates in the presence or absence of antifungals with serial concentration gradients.

For the XTT-reduction assay, biofilms formed from the *RAS10E* strain showed higher SMIC₅₀ values for fluconazole (at 12 h and 24 h biofilms), 5-fluorocytosine (at 6 h and 12 h biofilms), nystatin (at 6 h, 12 h and 24 h biofilms), itraconazole, amphotericin B, caspofungin and terbinafine (at 6 h, 12 h, 24 h and 36 h biofilms) than did those biofilms formed from the wild-type strain (Table 1). Furthermore, biofilms formed from *ras1Δ/Δ* showed lower SMIC₅₀ values for itraconazole (at 6 h, 12 h and 24 h biofilms), amphotericin B (at 12 h and 24 h biofilms), nystatin (at 6 h biofilms), fluconazole, caspofungin, terbinafine and 5-fluorocytosine (at the 6 h and 12 h biofilms), than did those bifilms formed from the wild-type strain (SN152) (Table 1). On the other hand, the SMIC₅₀ of the antifungals were increased with the mature of the biofilms formed from all the studied strains. As the biofilms grown for 36 h, the SMIC₅₀ of the antifungals against the biofilms were similar between that formed from the *ras1Δ/Δ* and the wild strain.

Table 1. The SMIC values of antifungals in *C. albicans* biofilms of mutant strains with and without farnesol

Farnesol		SMIC ₅₀ of Fluconazole (µg/ml)			
(µM)	pCaEXP-CAI4	RAS1OE	SN152	<i>ras1</i> Δ/Δ	
6 h					
0	>1024	>1024	128	4	
100	>1024	256	128	8	
200	>1024	128	64	4	
300	>1024	128	64	4	
12 h					
0	512	>1024	1024	128	
100	512	512	1024	128	
200	512	256	1024	128	
300	512	256	1024	1024	
24 h					
0	1024	>1024	1024	1024	
100	1024	1024	1024	1024	
200	512	512	1024	1024	
300	512	512	1024	1024	
36 h					
0	>1024	>1024	1024	1024	
100	>1024	>1024	1024	1024	
200	>1024	>1024	1024	1024	
300	>1024	>1024	1024	1024	
Farnesol		SMIC ₅₀ of Itraconazole (µg/ml)			
(µM)	pCaEXP-CAI4	RAS1OE	SN152	<i>ras1</i> Δ/Δ	
6 h					
0	>16	64	16	4	
100	>16	16	16	4	
200	>16	1	8	4	
300	>16	16	4	4	
12 h					
0	>16	128	>256	4	
100	>16	64	256	4	
200	>16	1	>256	4	
300	>16	64	4	4	
24 h					
0	>16	256	>256	>64	
100	>16	>64	>256	64	
200	>16	16	>256	16	
300	>16	64	>256	128	
36 h					
0	>16	256	>256	>256	
100	>16	>64	>256	>256	
200	0.125	32	>256	>256	
300	0.5	64	>256	>256	
Farnesol		SMIC ₅₀ of Amphotericin B (µg/ml)			
(µM)	pCaEXP-CAI4	RAS1OE	SN152	<i>ras1</i> Δ/Δ	
6 h					
0	1	4	8	8	
100	0.5	4	8	1	
200	1	4	4	4	
300	1	2	2	8	
12 h					
0	0.5	64	8	4	
100	0.5	8	4	4	
200	0.5	4	4	4	

300	0.5	16	2	2
24 h				
0	1	64	32	16
100	1	8	16	16
200	0.5	8	8	8
300	1	16	8	8
36 h				
0	0.25	64	32	32
100	0.25	8	2	2
200	0.125	16	2	2
300	0.125	16	2	2

Farnesol **SMIC50 of Caspofungin (µg/ml)**

(µM)	pCaEXP-CAI4	RAS1OE	SN152	<i>ras1Δ/Δ</i>
6 h				
0	0.125	1	16	4
100	0.0625	0.0625	0.25	0.25
200	0.0625	0.25	0.25	0.25
300	0.0625	0.0625	0.25	0.25
12 h				
0	>8	32	32	4
100	>8	0.125	0.5	0.5
200	0.25	0.25	0.5	1
300	0.25	0.0625	0.25	0.5
24 h				
0	>8	32	64	64
100	>8	16	0.5	0.5
200	>8	0.25	0.25	0.5
300	>8	2	0.5	0.5
36 h				
0	>8	32	>64	>64
100	0.25	0.125	1	0.5
200	0.125	0.5	0.5	0.5
300	0.0625	16	0.25	0.5

Farnesol **SMIC50 of Terbinafine (µg/ml)**

(µM)	pCaEXP-CAI4	RAS1OE	SN152	<i>ras1Δ/Δ</i>
6 h				
0	>256	512	64	16
100	>256	256	>512	>512
200	>256	256	>512	>512
300	>256	256	>512	>512
12 h				
0	>256	>512	512	64
100	>256	256	>512	>512
200	>256	256	>512	>512
300	>256	512	>512	>512
24 h				
0	>256	>512	>512	>512
100	>256	512	>512	>512
200	>256	>512	>512	>512
300	>256	512	>512	>512
36 h				
0	>256	>512	>512	>512
100	>256	64	>512	>512
200	>256	512	>512	>512
300	>256	512	>512	>512

Farnesol	SMIC50 of 5-Flucytosine (µg/ml)			
(µM)	pCaEXP-CAI4	RAS1OE	SN152	<i>ras1Δ/Δ</i>
6 h				
0	2	512	>512	256
100	1	4	512	128
200	2	2	512	256
300	16	16	512	128
12 h				
0	256	512	>512	128
100	1	1	512	512
200	4	4	16	64
300	16	0.5	512	512
24 h				
0	512	512	>512	>512
100	2	4	512	512
200	8	2	512	512
300	64	0.5	512	512
36 h				
0	512	512	>512	>512
100	0.5	0.5	512	512
200	16	2	512	512
300	64	64	512	512

Farnesol	SMIC50 of Nystatin (µg/ml)			
(µM)	pCaEXP-CAI4	RAS1OE	SN152	<i>ras1Δ/Δ</i>
6 h				
0	2	4	4	2
100	4	4	8	8
200	2	2	4	8
300	4	4	8	16
12 h				
0	4	8	4	4
100	4	4	8	8
200	2	2	4	8
300	4	4	16	32
24 h				
0	4	8	4	4
100	2	8	4	8
200	2	2	4	8
300	4	4	16	16
36 h				
0	8	8	4	4
100	4	8	8	8
200	2	2	4	8
300	0.5	1	16	16

Effects of farnesol on the susceptibility of the biofilms formed from the *RAS1* mutant strains to antifungals

Significant SMIC reductions were observed between farnesol-treated and untreated control when farnesol was added to the biofilms of the *RAS1OE* strains. Compared to the control, farnesol decreased the

resistance of *RAS10E* to fluconazole (at the 6 h, 12 h and 24 h biofilms), amphotericin B (at the 12 h, 24 h and 36 h biofilms), itraconazole, caspofungin, terbinafine, 5-fluorocytosine and nystatin (at the 6 h, 12 h, 24 h and 36 h biofilms). Moreover, the inhibitory effects of farnesol on the antifungal resistance (including fluconazole, itraconazole, amphotericin B, nystatin, caspofungin and 5-flucytosine) of *RAS10E* were more obvious than that on the wild-type strain (PCaEXP-CAI4) ($P<0.05$) (Table 1). In addition, the inhibitory effects of farnesol on the antifungal resistance of the biofilms from the *RAS10E* strain were in accordance to almost all of the growth phases.

In addition, compared to the control, farnesol decreased the resistance of the biofilms formed from *ras1Δ/Δ* to amphotericin B and caspofungin (at 6, 12, 24 and 36 h biofilms). However, farnesol did not decrease the resistance of the biofilms formed from *ras1Δ/Δ* to fluconazole, itraconazole and 5-fluorocytosine. On the other hand, farnesol increased the resistance of the biofilms formed from *ras1Δ/Δ* to the nystatin (at the 6h, 12, 24 and 36 h biofilms) and terbinafine (at the 6 and 12 h biofilms) (Table1). The inhibitory effects of farnesol on the resistance of *C. albicans* biofilm from the *RAS1*-deletion strain are not accordance at all the growth phase.

The morphologic changes of biofilms

Both CLSM and SEM observations showed that the inhibitory effects of farnesol on the hyphal growth of *RAS10E* were more obvious than that on the wild-type strain (Fig.3 and Fig.5), while the inhibitory effects of farnesol on hyphal growth of the *ras1Δ/Δ* were less obvious than those on the wild-type strain (Fig. 4 and Fig. 6). The CLSM showed that the biofilms of *RAS10E* and *ras1Δ/Δ* exposed to farnesol showed fewer hyphae but more pseudohyphae and blastospores than did those control without farnesol (Fig.3 C1D1, C2D2 and Fig. 4 G1H1, G2H2), respectively. In addition, the biofilms formed from *RAS10E* had more extensively grown hyphae and pseudohyphae with few blastospores than did those formed from the wild strain (pCaEXP-CAI4) (Fig. 3 A1C1, A2C2, A3C3, A4C4). Moreover, the biofilms formed from *ras1Δ/Δ* had fewer hyphae but more pseudohyphae and blastospores than did those formed from the wild strain (SN152) (Fig. 4 E2G2, E3G3).

A: farnesol-untreated pCaEXP-CAI4; B: farnesol-treated pCaEXP-CAI4; C: farnesol-untreated *RAS10E*; D: farnesol-treated *RAS10E*. A1, B1, C1 and D1 show 6 h *C. albicans* biofilms; A2, B2, C2 and D2 show 12 h *C. albicans* biofilms; A3, B3, C3 and D3 show 24 h *C. albicans* biofilms; A4, B4, C4 and D4 show 36 h *C. albicans* biofilms. The biofilms of C1C2 showed fewer hyphae but more pseudohyphae and blastospores than did those of D1D2. The inhibitory effects of C1D1, C2D2 were more obvious than did that of A1B1, A2B2. Magnification: 400×; farnesol: 200 μM.

E: farnesol-untreated SN152; F: farnesol-treated SN152; G: farnesol-untreated *ras1Δ/Δ*; H: farnesol-treated *ras1Δ/Δ*. E1, F1, G1 and H1 show 6 h *C. albicans* biofilms; E2, F2, G2 and H2 show 12 h *C. albicans* biofilms; E3, F3, G3 and H3 show 24 h *C. albicans* biofilms; E4, F4, G4 and H4 show 36 h *C. albicans* biofilms. The biofilms of G1G2 showed fewer hyphae but more pseudohyphae and blastospores than did those of H1H2. The inhibitory effects of G1H1, G2H2, G3H3, G4H4 were less obvious than did that of E1F1, E2F2, E3F3, E4F4. Magnification: 400×; farnesol: 200 μM

SEM analysis showed that the cells in farnesol-treated biofilms of *RAS1OE* and *ras1Δ/Δ* appeared in the short pseudohyphae and had a rough cell surface than did those in the biofilms without farnesol (Fig. 5 C1D1, C2D2, C3D3, C4D4 and Fig. 6 G1H1, G2H2, G3H3, G4H4). In addition, the biofilms formed from *RAS1OE* had more hyphae than did those formed from the control (pCaEXP-CAI4) (Fig. 5 A1C1, A2C2, A3C3, A4C4), while the biofilms formed from *ras1Δ/Δ* had fewer hyphae and more pseudohyphae than did those formed from the control (SN152) (Fig. 6 E2G2, E3G3, E4G4). The surface of the cells was similar between the biofilms of *RAS1OE* and *ras1Δ/Δ*.

A: farnesol-untreated pCaEXP-CAI4; B: farnesol-treated pCaEXP-CAI4; C: farnesol-untreated *RAS1OE*; D: farnesol-treated *RAS1OE*. A1, B1, C1 and D1 show 6 h *C. albicans* biofilms; A2, B2, C2 and D2 show 12 h *C. albicans* biofilms; A3, B3, C3 and D3 show 24 h *C. albicans* biofilms; A4, B4, C4 and D4 show 36 h *C. albicans* biofilms. The biofilms of C1-4 appeared in the short pseudohyphae and had a rough cell surface, while D1-4 appeared in the long hyphae and had smooth surfaces. The inhibitory effects of C1D1, C2D2 were more obvious than did that of A1B1, A2B2. Magnification: 2000×; farnesol: 200 μM

E: Farnesol-untreated SN152; F: farnesol-treated SN152; G: farnesol-untreated *ras1Δ/Δ*; H: farnesol-treated *ras1Δ/Δ*. E1, F1, G1 and H1 show 6 h *C. albicans* biofilms; E2, F2, G2 and H2 show 12 h *C. albicans* biofilms; E3, F3, G3 and H3 show 24 h *C. albicans* biofilms; E4, F4, G4 and H4 show 36 h *C. albicans* biofilms. The biofilms of G1-4 appeared in the short pseudohyphae and had a rough cell surface, while E1-4 appeared in the long hyphae and had smooth surfaces. The inhibitory effects of G1H1, G3H3, G4H4 were less obvious than did that of E1F1, E3F3, E4F4. Magnification: 2000×; farnesol: 200 μM

Farnesol decreased the expression of Ras1 in the biofilms formed from the *RAS1OE* strains

The results of RT-qPCR and western blotting showed that farnesol significantly reduced the expression of *RAS1* and the level of Ras1 in the biofilms formed by *RAS1OE* compared with those of the untreated controls at all studied phases ($P<0.05$) (Figs. 7 and 8). Farnesol also significantly reduced the expression of *RAS1* and the level of Ras1 in the biofilms formed by the wild-type strains compared with those of the untreated controls at all studied phases ($P<0.05$) (Figs. 7 and 8). Moreover, farnesol reduced the level of Ras1 and the expression of *RAS1* more obviously in the biofilms of the *RAS1OE* than that of the wild strains ($P<0.05$). Meanwhile, there was no expression of *RAS1* and Ras1 in the biofilms formed by *ras1Δ/Δ*, with or without farnesol.

Discussion

Previous investigations had clearly showed that *RAS1* is required for the induction of hyphal growth and the maintenance of the hyphal state [12, 14], and also has a close relationship with pathogenic processes [17], such as cell adhesion and biofilm formation. While no study reported the role of Ras1 in the antifungal resistance of *C. albicans* and the relevant farnesol regulation mechanism of the biofilms, which were discussed in this study.

Our study found that the *RAS1* involved in the antifungal resistance of *C. albicans*. For the *C. albicans* in planktonic form, *RAS1*-overexpressing strain increased resistance of planktonic *C. albicans* to fluconazole and itraconazole, and the *RAS1* deletion strain reduced resistance to all the studied antifungals. For the *C. albicans* in biofilm form, the *RAS1* overexpression increased the resistance of the biofilms to the fluconazole, itraconazole, amphotericin, nystatin, caspofungin, terbinafine, and 5-fluorocytosine, and *RAS1* deletion reduced the resistance of those above antifungals. The findings indicated that *RAS1* is associated with the antifungal resistance of *C. albicans* in planktonic and biofilms forms.

Currently, azoles, polyenes, echinocandins and miscellaneous antifungals are the main antifungal families used in clinical practices. In this study, the antifungal susceptibilities of the studied strains were analyzed for the main antifungals including fluconazole, itraconazole, amphotericin B, nystatin, caspofungin, terbinafine and 5-fluorocytosine. Fluconazole/itraconazole impairs ergosterol synthesis and leads to a cascade of membrane abnormalities in the fungus [18]. Amphotericin B/nystatin acts through pore formation at the cell membrane and induces the ergosterol sequestration after binding to ergosterol [19]. Caspofungin blocks the synthesis of β -(1,3)-D-glucan, an essential component of the fungal cell wall [20]. Terbinafine inhibits the ergosterol synthesis and preventing the conversion of squalene to lanosterol by inhibiting squalene epoxidase [21]. 5-fluorocytosine works by being converted into 5-fluorouracil inside the fungal, and blocks its ability to make protein [22]. These antifungal drugs have different mechanisms of action, then the effects of antifungals to the mutant strains might be complex. The results that *RAS1* overexpression increased its susceptibility, and the *RAS1* deletion decreased its susceptibility, to multiple antifungals with different mechanisms, implies that *RAS1* might has a non-specific mechanism in regulating the resistance action of *C. albicans* against those antifungals.

In this study, farnesol decreased the expression of *RAS1* gene and the level of Ras1 protein in *C. albicans* biofilm formed from the *RAS1*-overexpressing strain and the wild strain. Meanwhile, the inhibitory effects of farnesol on the resistance of *C. albicans* biofilm from the *RAS1*-overexpressing strain were more obvious than that formed from the wild-type strain (PCaEXP-CAI4) and the *RAS1*-deletion strain. The results suggested that *RAS1* involve in the antifungal resistance regulated by farnesol. On the other hand, the effects of farnesol on the resistance of *C. albicans* biofilm from the *RAS1*-deletion strain are not closely accordance to that from the *RAS1*-overexpressing strain, farnesol decreased the resistance of the biofilms from *RAS1*-deletion strain to amphotericin B and caspofungin, while increased that to the nystatin and terbinafine. The possible reason for the results might be the antifungal resistance change of the *C. albicans* biofilms following the *RAS1* deletion, and combined with the complexity of the multiple antifungals having different mechanisms, which caused the different results of the antifungal resistance of this strain.

Morphological observations in this study showed that the *RAS1*-overexpression increased the hyphal growth and the *RAS1*-deletion decreased the hyphal growth of the *C. albicans* biofilms. Previous studies showed that *RAS1* deletion mutants are impaired in hyphal development under many different induction conditions [23]. The morphological observations of the deletion strain in the present study were in accordance with the previous study. Furthermore, both CLSM and SEM observations showed that the

inhibitory effects of farnesol on *RAS1*-overexpression were more obvious than that on the wild-type strain, and the inhibitory effects of farnesol on *RAS1*-deletion strain were less obvious than those on the wild-type strain. Combined with the results that farnesol increased the susceptibility of those biofilms to most antifungals, the morphological results suggested that farnesol decrease the hyphae growth through regulating the *RAS1* expression of the biofilms, and involve in the antifungal resistance of *C. albicans* biofilms subsequently.

The formation of mature biofilms was composed of a series developed steps. The formation of *C. albicans* biofilms started with the initial adherence of yeast cells (0-2 h), followed by germination and micro-colony formation (2-4 h), filamentation (4-6 h), monolayer development (6-8 h), proliferation (8-24 h), and maturation (24-48 h) [24]. In this study the main stages of biofilm were investigated to clarify the effects of farnesol on *RAS1* in *C. albicans* biofilms. A phase-specific mechanism might be involved in biofilm resistance, which appeared of the increased resistance of *C. albicans* with the mature biofilms (24h and 36 h). Because of this phase-specific increased resistance, the inhibitory effects of farnesol were reduced with the mature of biofilms. In addition, the study showed that the inhibitory effects of farnesol on Ras1 level and the antifungal resistance of the biofilms from the *RAS1*-overexpressing strain were in accordance to almost all of the growth phases, which indicated that *RAS1* involved in the antifungal resistance caused by farnesol.

On the other hand, the effects of farnesol on the resistance of *C. albicans* biofilm from the *RAS1*-deletion strain are not accordance at the different growth phase. The possible reason of this might be the strain deleted *RAS1* caused the resistance change of itself, a phase-specific mechanism involved in the resistance of biofilm, and other complicated and multiple factors affected the resistance of the biofilms of the *RAS1*-deletion strain, which need to be further discussed.

In conclusion, our data indicate that *RAS1* involved in the resistance of *C. albicans* biofilms, and also involved in the inhibitory effects of farnesol on the resistance of *C. albicans* biofilms. To the best of our knowledge, no previous studies reported that *RAS1* is associated with the resistance of *C. albicans* biofilms, and also no study of the *RAS1* responding to farnesol associated with its antifungal effectiveness or capacity. In addition, the observations that *RAS1* deletion or over-expression, with or without farnesol, affected susceptibility to multiple antifungals with different mechanisms of action implies a non-specific effect. Additional research involving the specific molecular mechanisms should be further pursued to elucidate the antifungal resistance mechanisms of *C. albicans* biofilms.

Conclusion

The extensive use of antifungals has led to the increased resistance of *C. albicans* biofilms to the few existing antifungals. Farnesol enhances the susceptibility of *Candida albicans* biofilms to antifungals, while the mechanisms of this behavior are poorly understood. Thus, it is crucial to explore the mechanisms of preventive strategies for the farnesol-mediated increase in susceptibility to various antifungals targeting biofilm-related infections. *RAS1* regulated the hyphal growth of *C. albicans* in the

previous reports. For the first time, this study demonstrated that Ras1 involved in the antifungal resistance of *Candida albicans*, and also involved in the inhibition of farnesol on the resistance of biofilms.

Methods

Strains, plasmids and media

The strains and plasmids used in the study are listed in Table 2. The Wild-type clinical isolate SC5314 (American Type Culture Collection, America) and the resistant strain were used to identify the effects of farnesol on the RAS1 expression. The resistant strain derived from SC5314 induced via the serial fluconazole concentration gradient method until the MIC reached or exceeded 64 µg/ml. *RAS1* overexpressing strain (*RAS1OE*) was generated from CAI4 (William A. Fonzi, Department of Microbiology and Immunology, Georgetown University, Washington, USA) using *RAS1*-pCaEXP plasmids. The wild-type strain PCaEXP-CAI4 was generated from CAI4 using the plasmid pCaEXP (BioVector NTCC Inc., China). *RAS1* deletion strain (*ras1Δ/Δ*) was generated from SN152 (School of Pharmacy, The Second Military Medical University). Fusion PCR was performed to create disruption fragments of *C. albicans HIS1* and *LEU2* coding sequences, and the *HIS1* and *LEU2* disruption fragments were transformed into SN152 to obtain *ras1Δ/Δ* [25].

Freshly grown yeast cells from Sabourd's Dextrose Agar (SDA) plates were incubated in yeast peptone dextrose (YPD) medium and grown overnight in an orbital shaker at 30°C. The cells were collected and resuspended in RPMI 1640 supplemented with L-glutamine and buffered with morpholine propane sulfonic acid (Gibco Ltd., Paisley, U.K.). The solution was adjusted to a cell density of 1×10⁶ cells/ml for the experiments. All experiments were performed in triplicate on three separate occasions.

Table 2. Strains and plasmids used in this study.

Strains	Genotype and Description ^a	Reference
<i>C. albicans</i> strains		
SC5314	Wild-type clinical isolate	American Type Culture Collection, America
Resistant strain	derived from SC5314 induced via the serial fluconazole concentration gradient method until the MIC reached or exceeded 64 µg/ml	Yu LH et al. 2011 Xia J et al. 2017
CAI4	<i>ura3::λimm434/ura3::λimm434</i> <i>URA3</i> auxotrophic strain	Fonzi and Irwin (1993)
PCaEXP-CAI4	<i>ura3::λimm434/ura3::λimm434</i> -(pCaEXP <i>URA3</i>) Wild-type strain transformed with pCaEXP used as a control of overexpression experiments	In this study
RAS1OE	<i>ura3::λimm434/ura3::λimm434</i> -(RAS1-pCaEXP <i>RAS1</i> , <i>URA3</i>) <i>RAS1</i> -overexpressing strain	In this study
SN152	<i>arg4/arg4 leu2/leu2 his1/his1 URA3/ura3::imm434</i> <i>IRO1/iro1::imm434</i> Arg4, Leu2, His1 auxotrophic strain	Noble SM et al. (2005)
<i>ras1Δ/Δ</i>	<i>arg4/arg4 leu2/leu2 his1/his1 URA3/ura3::imm434</i> <i>IRO1/iro1::imm434</i> <i>ras1::C.d.HIS1/ras1::C.m.LUE2</i>	In this study
Plasmids		
pCaEXP	<i>URA3</i> and <i>MET3</i> promoter integrating plasmid	R. S. Care et al. (1999)
RAS1-pCaEXP	Constructed by integration of <i>RAS1</i>	In this study
pSN52	With <i>HIS1</i> marker	Noble SM et al. (2005)
pSN40	With <i>LUE2</i> marker	Noble SM et al. (2005)

^a *C.d.*, *C. dubliniensis*; *C.m.*, *C. maltose*

Biofilm formation and farnesol treatment

Standardized suspensions of the strains were dispensed into flat-bottom microtiter dishes (Corning, Inc., N.Y., USA). The cells were incubated at 37°C in a moist chamber with 5% CO₂. After 2 h of incubation, non-adherent cells were removed by thoroughly washing the biofilms three times with PBS. Then, the biofilms were formed depending on the selected time periods (6, 12, 24 and 36 h).

For the farnesol treatment, equal volumes of farnesol (100-300 μ M) or sterile water (with an equal concentration of methanol) were added to the farnesol-treated and untreated control groups, respectively. Stock solutions (100 mM) of farnesol (E, E farnesol; Sigma Chemical Co., St. Louis, Mo) were dissolved in 100% (vol/vol) methanol and frozen at -70°C until use. The effect of methanol on the growth of *C. albicans* was evaluated by testing different methanol concentrations without antifungals, ranging from 0.05–1% (v/v) [26]. The highest methanol concentration used in the microdilution plates was 0.1% (v/v), which presented no antifungal activity.

Susceptibility tests

The antifungals used in the tests were fluconazole (Sigma-Aldrich, St Louis, MO, USA), itraconazole (Selleckchem, Houston, TX, USA), amphotericin B (Sigma-Aldrich, St Louis, MO, USA), caspofungin (Sigma-Aldrich, St Louis, MO, USA), terbinafine (Selleckchem, Houston, TX, USA) and 5-flucytosine (Selleckchem, Houston, TX, USA), nystatin (Selleckchem, Houston, TX, USA).

The susceptibility of *RAS1* mutant strains in planktonic form to antifungals was determined using a spot assay [27]. The mutant and the wild-type strains were incubated in YPD medium and grown for 16 h in an orbital shaker at 30°C. The yeast cells were harvested during the logarithmic growth phase, washed twice with PBS and suspended in fresh medium to an OD_{600nm} of 1.0. Then, 5 μ l of tenfold serial dilutions of the suspensions were spotted onto YPD plates in the presence or absence of antifungals with serial concentration gradients. Antifungals were added to the medium at a concentration of 4 μ g/ml for fluconazole, 0.5 μ g/ml for itraconazole, 2 μ g/ml for amphotericin B, 0.5 μ g/ml for caspofungin, 15 μ g/ml for terbinafine, 8 μ g/ml for 5-fluorocytosine and 2 μ g/ml for nystatin. Growth differences were measured after incubation at 30°C for 48 h.

The susceptibility of *RAS1* mutant strains in biofilm to antifungals was determined using the XTT [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide]-menadione (0.5 mg/ml XTT, 1 μ M menadione) reduction assay (Sigma-Aldrich, St Louis, MO, USA) [28]. Colorimetric changes were analyzed in a microtiter plate reader (BioTek Instruments, Inc., VT, USA) at a wavelength of 490 nm, which measured the changes in the metabolic activity of the biofilms [29]. The biofilms were incubated as described above. A series of antifungal agent-free wells and biofilm-free wells were also included to serve as the positive and negative controls. Drugs were prepared in serial 2-fold dilutions, and their final concentrations ranged from 4 to 1024 μ g/ml for fluconazole, 1 to 256 μ g/ml for itraconazole, 0.25 to 64 μ g/ml for nystatin, amphotericin B and caspofungin, and 2 to 512 μ g/ml for terbinafine and 5-flucytosine. Antifungals were added to the biofilms and incubated for an additional 24 h at 37°C. The value of the background OD measurement, obtained from biofilm-free wells processed in the same manner as the inoculated wells, was subtracted from the OD measurement of each well. After this subtraction, the percentage of growth in each well was calculated as the OD of each well/OD of the drug-free well. The lowest drug concentrations that inhibited biofilm growth by 50% were considered the sessile MIC₅₀ (SMIC₅₀) of this antifungal.

The morphological observations using CLSM and SEM

Biofilms formed from the mutant strains, the wild-type strains, and the farnesol-treated strains were formed on the glass bottom of cell culture dishes for morphological observation. For CLSM observation, the formed biofilms were fixed with 4% paraformaldehyde overnight, washed with PBS and stained with 500 µl calcofluor white stain (0.0025 g/ml; Sigma Chemical Co., St. Louis, MO) [30] for 30 min at 37°C in the dark. The biofilms were observed using a Zeiss LSM700 microscope with a video capture system, automatic camera, image analysis hardware and software (Carl Zeiss, Inc., Oberkochen, Germany), and a 488 nm argon ion laser. For SEM observation, the formed biofilms were fixed with 2.5% glutaraldehyde solution overnight. The biofilms were subsequently washed twice with distilled water, dehydrated in an ethanol series (70% for 10 min, 95% for 10 min and 100% for 20 min) and air-dried overnight in a desiccator. Then, the biofilms were metalized by gold sputtering for 45 s in a High Vacuum Evaporator, followed by bonding to carbon double-side tape and processing for SEM (FEI Inc., Hillsboro, USA).

Ras1 of the biofilms regulated by farnesol via RT-qPCR and confirmed by Western blot analysis

RNA samples of the farnesol-treated and untreated control biofilms were purified using a modified hot phenol method [31]. The cDNA was synthesized using the reverse transcription system (TaKaRa, Bio Co., Ltd., Dalian, China) and performed on an ABI 7300 Fast real-time PCR machine (Applied Biosystems, Rotkreuz, Switzerland) using qPCR SYBR Green Mix (Thermo Scientific, Waltham, MA, USA). The PCR program used was as follows: activation at 95°C for 10 min; 40 cycles of amplification (95°C for 15 s, 55°C for 1 min); 70°C for 20 s; cooling at 4°C. After amplification, a melting curve analysis was performed to ensure the absence of primer dimers. The expression of genes was calculated using the $2^{-\Delta\Delta C_t}$ method [11]. *ACT1* was used as a reference gene. Primers were all designed by Shanghai Generay Bio-Tech Co., Ltd. (Table 3).

Total protein extracts were prepared as described for *C. albicans* biofilms using an immunoprecipitation protocol [32]. Protein concentrations were determined using a BCA Protein Assay Kit (Sigma-Aldrich, St. Louis, MO, USA). A total of 15 µg protein diluted in 6× loading buffer was separated on a 10% SDS-PAGE gel and then blotted onto PVDF membranes (Millipore, MA, USA). Membranes were blocked in 0.01 M PBS containing 5% skim milk and 0.1% Tween-20 at room temperature, followed by incubation of membranes overnight with the primary antibodies (Ras1, 1:1000 dilution; GAPDH, 1:8000 dilution; Bioworld, MN, USA) at 4°C. Membranes were then rinsed with PBST (0.01 M PBS plus 0.1% Tween-20) and incubated with secondary antibodies (1:10000 dilution, Bioworld, MN, USA) at room temperature for 1 h. Membranes were visualized with Super Signal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific Inc., Rockford, IL, USA) and exposed to Kodak X-ray films. GAPDH was used as an internal control in the experiments.

Table 3. Sequences of primers

Gene	Sequences
<i>RAS1</i>	F: GGTAATCCGCTTTAACCATTC R: GCCAGATATTCTTCTTGTCCAG
<i>ACT1N</i>	F: GCCGGTGACGACGCTCCAAGAGCTG R: CCGTGTTCAATTGGGTATCTCAAGGTC

Statistical analysis

All quantitative experiments were performed in triplicate for statistical analyses. Data were analyzed using SPSS19.0 software (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) were used to analyze the differences between the groups, while a paired t-test was performed for intragroup comparisons. The rank sum test was used to analyze ranked data. Comparisons resulting in *P* values of less than 0.05 were considered statistically significant.

List Of Abbreviations

*RAS1*OE: *RAS1* overexpressing strain; CLSM: confocal laser scanning microscope; SEM: scanning electron microscopy; YPD: yeast peptone dextrose; ANOVA: One-way analysis of variance.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

XW conceived the study, participated in study design and data analysis and was responsible for writing and submitting the final manuscript. LLZ structured the *RAS1* overexpressing and deletion strains and performed statistical analysis and drafted the manuscript. SYC carried out the experimental studies related to the XTT reduction assay, spot assay. ZZZ participated in RT-qPCR and western blot analysis. LLJ participated in morphological observations by CLSM and SEM. All authors read and approved the manuscript.

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Figures

Figure 1

RAS1 and Ras1 regulated by farnesol via RT-qPCR and western blot

Figure 2

Susceptibility tests of the RAS1 mutant strain of planktonic *C. albicans*

Figure 3

The morphological changes in 6 h, 12 h, 24 h and 36 h *C. albicans* biofilms of RAS10E shown by CSLM.

Figure 4

Morphological changes in 6 h, 12 h, 24 h and 36 h *C. albicans* biofilms of *ras1* Δ/Δ shown by CSLM.

Figure 5

The morphological changes in 6 h, 12 h, 24 h and 36 h *C. albicans* biofilms of RAS10E shown by SEM.

Figure 6

The morphological changes in 6 h, 12 h, 24 h and 36 h *C. albicans* biofilms of *ras1Δ/Δ* shown by SEM.

Figure 7

The expression of the RAS1 gene in farnesol-treated RAS1OE

Figure 8

The expression of the Ras1 protein in RAS1OE and pCaEXP-CAI4