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Effects of environmental factors on dimorphic transition of the jelly mushroom *Tremella fuciformis*

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Abstract. – *Tremella fuciformis*, a typical dimorphic fungus, displays two distinct morphological transitions: (i) yeast-pseudohyphae transition; (ii) yeast-hyphae transition. To understand the mechanism of its dimorphism, we initiated a study by investigating how environmental factors affect the yeast-to-pseudohypha transition, including pH value, temperature, carbon source, nitrogen source, cultural time, ethanol and inoculum size. Our studies showed that the optimal conditions for yeast-to-pseudohypha transition were as follows: incubating at 25 to 30°C for 3 to 7 days in media containing glucose as the carbon source, ammonium as the nitrogen source and 10 ml/L ethanol. Below a threshold of inoculum size (10^5 cells/L), the transition ratio fluctuated markedly, which made us propose that quorum sensing molecules contribute to the dimorphic behavior in *T. fuciformis*.

Inoculum size / Morphology / Pseudohypha / Tremellales / Yeast

INTRODUCTION

Fungal dimorphism is the morphological interconversion between a unicellular yeast phase and a multicellular filamentous phase (hyphae or pseudohyphae) which are generally controlled by environmental and other factors (Rappleye and Goldman, 2006). A variety of fungi have been reported to be able to undergo this morphological transition (Klein & Tebbets, 2007; Nadal *et al.*, 2008; Nemecek *et al.*, 2006). However, the analysis of dimorphism was mainly based on the model organism Saccharomyces cerevisiae Meyen ex E. C. Hansen and some human or plant pathogenic fungi, where cell differentiation is linked to pathogenecity (Cervantes-Chávez & Ruiz-Herrera, 2006; Enczi, 2007). Here we used the edible fungus, *Tremella fuciformis* Berk., for a study on fungal dimorphism.

Tremella fuciformis, or white jelly mushroom, is a typical mycoparasitic and dimorphic fungus with great nutritional and medicinal value (Bandoni & Boekhout, 1998; Huang, 2000). It displays both yeast to pseudohyphae and yeast to hyphae transitions. In the former case, haploid, budding, yeast-like basidiospores elongate with daughter cells remaining connected with their mother-cells resulting in string-like pseudohyphae. In the latter case, haploid cells of opposite mating

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types fuse and give rise to the dikaryotic hyphae. Then the dikaryon may switch back to the yeast form, which is also known as arthrospores (Liu *et al.*, 2007).

Dimorphism behavior is affected by many regulators including environmental factors, e.g. carbon source, nitrogen source, C/N ratio, pH value, temperature, cultural time, gene expression and signal pathways. Roles of environmental factors in dimorphism have been well documented in several fungi. Cao *et al.* (2007) reported the respective effects of temperature, pH value, and salinity on the growth and dimorphism of the human pathogen *Penicillium marneffei* Segretain. Scheld & Perry (1970) demonstrated that the degradation products of sugar have a marked effect on morphogenesis of the mushroom *Lenzites saepiaria* (Wulf. ex Fr.) Fr. The smut fungus *Ustilago maydis* (DC.) Corda was found to be more likely to grow in mycelial form in the presence of lipid (Klose *et al.*, 2004) and at acid pH (Ruiz-Herrera *et al.*, 1995). Nonetheless, the environmental factors act on dimorphism together with signal pathways and control of gene expression. Ethanol was confirmed to serve as an inducer of pseudohyphal transition in the yeast *Candida tropicalis* (Castell.) Berkhout via phosphoinositide signaling (Suzuki *et al.*, 2006). The yeast *S. cerevisiae* undergoes a dimorphic transition when confronted with nutrient limitation, and this transition process is governed by at least three signaling pathways (Truckses *et al.*, 2004). There, dimorphism-related genes and proteins regulated by environmental factors have been identified and characterized. *STE11* disruption revealed a central role of a MAPK pathway in dimorphism and mating in the yeast *Yarrowia lipolytica* (Wick., Kurtzman & Herman) Van der Walt & Arx as well (Cervantes-Chávez & Ruiz-Herrera, 2006), and genes encoding ammonium permeases were found to be involved in ammonium signaling for dimorphism transition in several fungi (Smith *et al.*, 2003). Wolff *et al.* (2002) characterized a series of key genes involved in the control of dimorphism (*pkaR* and *pkaC* encoding the regulatory and catalytic subunit) in the zygomycete *Mucor circinelloides* Tiegh.

However, little is known about dimorphism-related factors in *Tremella fuciformis.* In order to understand how environmental factor, gene expression and signal pathway co-regulate the dimorphism behavior in *T. fuciformis*, we initiated the study by investigating effects of environmental factors on yeast-to-pseudohyphae transition, including pH value, temperature, carbon source, nitrogen source, cultural time, ethanol and inoculum size.

MATERIALS AND METHODS

Strains. Single spore isolates T3 and T8, two compatible haploid strains, were isolated from basidiospores of *T. fuciformis* and maintained at room temperature on PDA (Difco, America) slant.

Growth medium. The designed liquid medium was autoclaved at 121°C for 30 min containing the following per liter of distilled water: carbon source, 20 g; nitrogen source, 20 mM; $MgSO_4 \cdot 7H_2O$, 0.25 g; $KH_2PO_4 \cdot 3H_2O$, 0.5 g; $ZnSO_4 \cdot 7H_2O$, 2 mg; $CaCl_2 \cdot 2H_2O$, 0.5 g. Five different carbon sources (sucrose, glucose, maltose, mannitol and lactose) and nitrogen sources (potassium nitrate, ammonium nitrate, ammonium sulfate, ammonium tartrate and glycine) were tested. The pH of the medium was adjusted by the addition of dibasic sodium phosphate-citric acid

buffer to the desired pH value (3.0, 4.0, 5.0, 6.0, 7.0 and 8.0). All assays were repeated three times to ensure reproducibility of the results.

Preparation for inoculum and culture condition. For the preparation of inocula, cells maintained on PDA slants were first transferred to the 50 ml liquid media in 250 ml flask and incubated at 25°C on an orbital shaker (Fuma, China) at 150 rpm for 3~5 d. The cultures were centrifuged at 12,000 rpm for 15 min, washed three times with distilled water, resuspended in distilled water and stored at 4°C until use as inocula. 10^5 cells/L were incubated in 250 ml flasks containing 50 ml of the designed growth media and incubated at 25°C (except the experiment of incubated temperature) on an orbital shaker at 150 rpm for 3 to 5 d (except the experiment of incubated time). To identify the incubated temperature and time at which the conversion of yeast to pseudohyphae occurs, we chose 20°C, 25°C, 28°C, 30°C and 37°C as the temperature parameters and 3, 5, 7, 9 and 11d as the time parameters. In order to confirm the effect of ethanol and inoculum size on dimorphic transition, 5, 10, 15, 20 and 25 ml/L ethanol, concentration of 10^3, 10^4, 10^5, 10^6 and 10^7 cells/L were prepared in 50 ml liquid media respectively.

Morphology observation. Cells were observed with a DME microscopy (Leica, Germany). Photos of cell morphology were captured with a Panasonic colour CCTV Camera. In our study, pseudohyphae were defined by the following criteria: (i) three or more rounded or swollen cells connected at the end of the long axes or in a definite direction; (ii) an elongated cell with a round or ellipsoidal daughter cell and an ellipsoidal cell having two branched daughter cells. In contrast, if only two cells were connected, these were regarded as yeast cells (Suzuki *et al.*, 2006). For morphology analysis, at least 300 cells were scored under the microscopy.

RESULTS

Effect of pH and temperature on dimorphic transition of *T. fuciformis*.

Both *T. fuciformis* strains T3 and T8 were cultured in liquid basic media at pH value ranging from pH3 to pH8 (Fig. 1A). Microscopic examination revealed transition from yeast to pseudohyphae at weak acidic and neutral condition. For T3, pH6 was the optimum to induce transition, with a transition ratio up to 17.70%. When cultured at strong acidic or base condition, only weak transition ability of 2.40% at pH3 and 3.13% at pH8 were found for T3. A similar tendency was also found for T8, with the only difference being that the optimal pH value was pH5.

For temperature, 25°C was the optimal temperature to induce yeast to pseudohyphae transition for both T3 and T8 (Fig. 1B). At higher temperatures of 28 and 30°C, a lower transition rate was observed while no marked morphological switch was observed at 20 or 37°C.

Effect of carbon and nitrogen source on dimorphic transition of *T. fuciformis*.

Of the five carbon sources tested, the highest transition rates were found on glucose at 20.67% and 24.92% for T3 and T8, respectively (Fig. 1C).

The effect of nitrogen source was strongest with ammonium nitrate at 25.21% and 20.35% for T3 and T8, respectively, and weakest on glycine at 1.16% and 2.22% for the two respective strains (Fig. 1D).

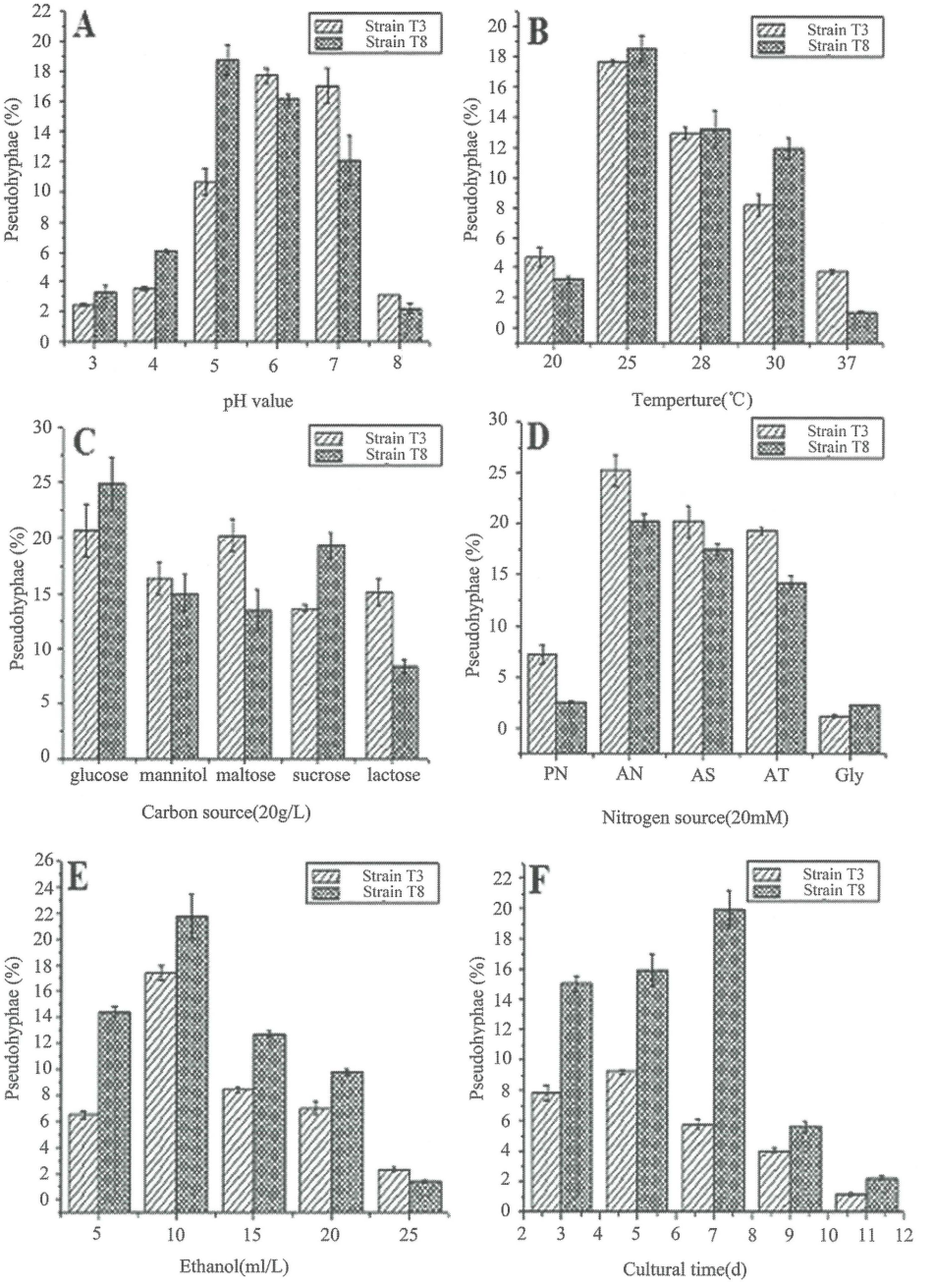

Fig. 1. Effects of pH (A), temperature (B), carbon source (C), nitrogen source (D), ethanol (E) and time (F) on the dimorphic transition of *T. fuciformis*. Abbreviations in D: PN, potassium nitrate; AN, ammonium nitrate; AS, ammonium sulfate; AT, ammonium tartrate; Gly, glycine. The error bars reflect standard deviation.

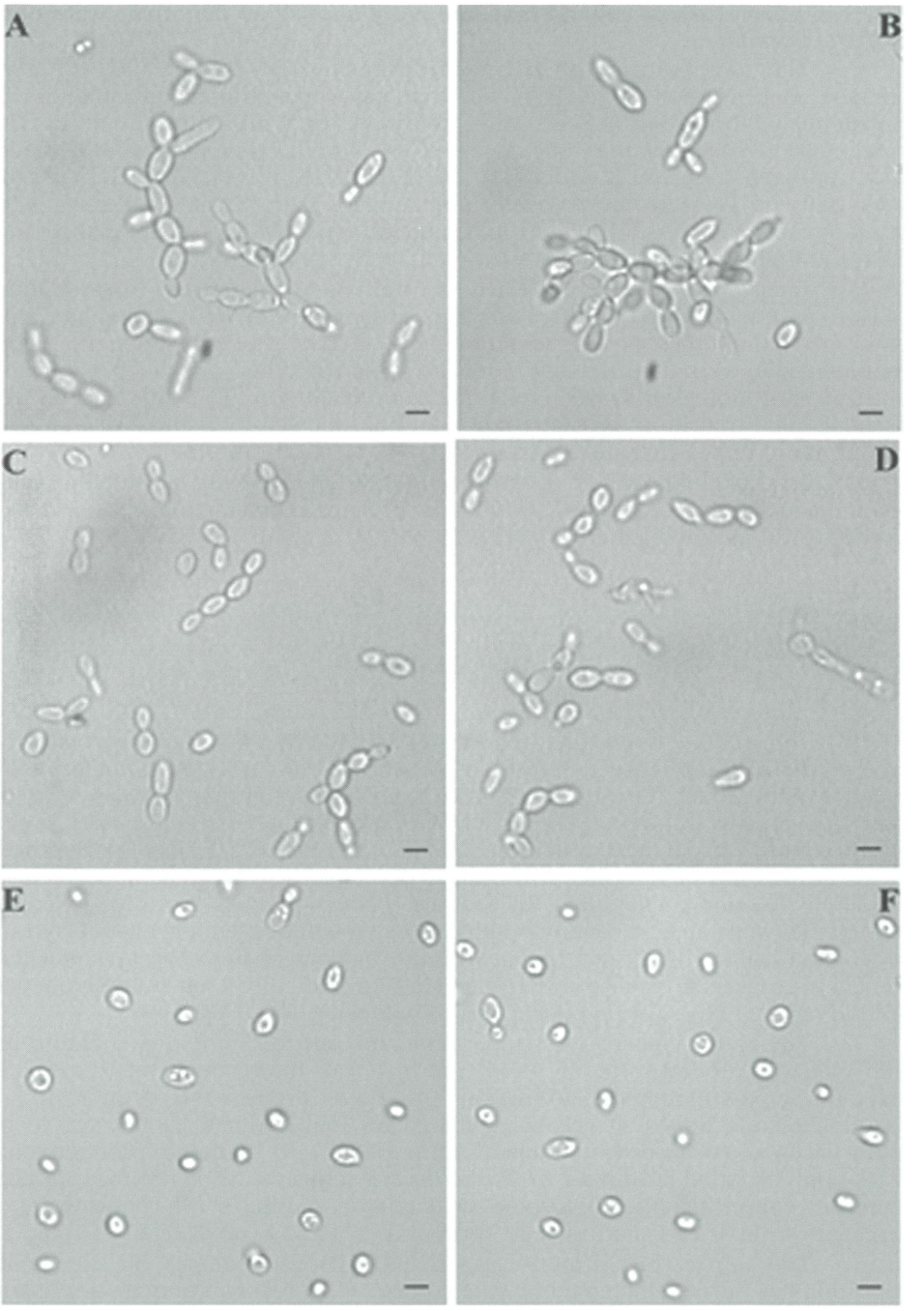

Fig. 2. Influence of inoculum size on the pseudohyphal transition of *T. fuciformis*. (A), (C) and (E) represented different cell morphology of T3 when 10^3, 10^5 and 10^7 cells/L incubated in PDB, respectively; (B), (D) and (F) represented different cell morphology of T8 when 10^3, 10^5 and 10^7 cells/L incubated in PDB, respectively. Scale bar, 10 μm.

Effect of ethanol stress, cultural time and inoculum size on dimorphic transition of *T. fuciformis.*

The morphologies of cells were characterized in media of different ethanol concentration (Fig. 1E). The optimal concentration of ethanol for pseudohyphal transition was 10 ml/L, followed by 5 ml/L and 15 ml/L. The percentage of pseudohyphae was negligible in 25 ml/L ethanol-containing media with approximately 2.28% and 1.41% for T3 and T8, respectively. Besides, the yeast cells of T8 were more sensitive to ethanol than T3. The proportion of pseudohyphal cells was 14.41% (5 ml/L ethanol), 12.68% (15 ml/L ethanol) and 9.74% (20 ml/L ethanol).

Incubation time also affected the morphogenetic switch from budding yeast to pseudohyphae (Fig. 1F). After 5 d, T3 showed 9.25% pseudohyphae, with other incubation times showing weaker effects. After 11 d of cultivation, no visible pseudohyphae were detected any more. T8 is generally had a higher tendency to undergo morphological switch under the same condition. The optimal time for pseudohyphal transition for that strain was 7 d, followed by 3 and 5 d. For both strains, more than 9 d resulted in a decreased transition ratio (5.62%).

Two haploid cells of *T. fuciformis* developed mostly as budding yeast when inoculated at $> 10^5$ cells/L, while at $\leq 10^5$ cells/L inoculation, cells switched to the typical pseudohyphal form (Fig. 2).

DISCUSSION

Tremella fuciformis dimorphism was affected by the tested environmental factors, all of which induced transition of haploid yeast cells of *T. fuciformis* to pseudohyphal growth. Previous studies have shown that pH plays a key role in the transition process. However, the effect of pH value on different species varies. When cultured under acid condition, *Candida albicans* (C. P. Robin) Berkhout grows in the form of yeast-like cell while it develops pseudohyphae under neutral conditions (Konno *et al.*, 2006). In contrast, *Ustilago maydis* grows yeast-like at neutral pH, while an acid condition induces the growth of pseudohyphae. Contrary to the long-standing belief that *U. maydis* is the only fungal dimorphic system where an acid pH triggers mycelial growth (Ruiz-Herrera *et al.*, 1995), our study shows that an acid pH also favors the production of pseudohyhae for *T. fuciformis*.

The optimal incubation temperature and time for *T. fuciformis* dimorphic transition were found to be 25 to 30°C at 3 to 7 d. Interestingly, after a steady increase in pseudohyphae formation for both strains, the proportion of pseudohyphae dropped sharply if incubated for longer times. T8 seemed to be more inclined (with nearly doubled ratio of T3) to undergo pseudohyphal transition. Different from that of *T. fuciformis*, temperature is the most critical factor in controlling the dimorphic switch in *P. marneffei* which grows as a mycelium form at 25°C and as yeast form at 37°C (Cao *et al.*, 2007).

The impact of different carbon and nitrogen sources on dimorphic transition has been well studied in other species. Our results are quite consistent with those reported. Ruiz-Herrera *et al.* (1995) reported that glucose is the most effective inducer for the dimorphic switch in *U. maydis*, while high concentrations (5-10%) again reduced mycelial growth. Thus, we maintained the concentration of carbon sources at 2%. The mechanism of how different concentrations affect the

dimorphic transition remains to be discovered. For nitrogen source, we found that ammonium salts were the optimal inducers of yeast-pseudohyphae transition, which is in agreement with what Ruiz-Herrera *et al.* have found (Ruiz-Herrera *et al.*, 1995).

Suzuki *et al.* (2006) could show that ethanol contributes to dimorphism of *C. tropicalis*, and best results were obtained when ethanol was added at a concentration of 25 ml/L. In contrast to *C. tropicalis*, we observed that 10 ml/L is the optimum concentration for *T. fuciformis*. In agreement with Suzuki *et al.*, we suggest that some changes in cell membrane occurred when we added ethanol and changes in membrane may influence some signal transduction pathways, such as MAPK, PKA and AMPK, which have proved to regulate the dimorphism in *S. cerevisiae* (Truckses *et al.*, 2004).

For *T. fuciformis*, inoculum size is another contributor to dimorphism. Both haploid strains developed as budding yeast when inoculated at $> 10^5$ cells/L, while cells produced typical pseudohyphal morphology when inoculated at $\leq 10^5$ cells/L. Similar results have been obtained in other species, such as *C. albicans* (Nikerson *et al.*, 2006) and *Ceratocystis ulmi* (Buisman) C. Moreau (Hornby *et al.*, 2004). Both of them were confirmed to show the phenomenon of density dependence which is indicative of an active quorum sensing system. Extracellular cell density-dependent signals, quorum sensing molecules (QSMs) have been identified to be farnesol and tyrosol for regulating dimorphism. Farnesol inhibits the morphological transition from budding yeast to the hyphal form at high cell densities in *C. albicans* (Hornby *et al.*, 2001), while tyrosol accelerates the development of this transition (Alem *et al.*, 2006; Chen *et al.*, 2004). As suggested by Chen *et al.* (2004), different QSMs co-regulate dimorphism in *C. albicans*. Besides, the addition of farnesol led to a dramatic decrease of intracellular cAMP levels, an important intracellular second messenger (Hornby *et al.*, 2001). Thus, it can be presumed that QSMs play a key role in signaling transduction. However, so far, quorum sensing in *T. fuciformis* has not been reported. To investigate QSMs and their impact on dimorphism, the purification from extracelluar supernatant is in progress to find out whether similar QSMs exist in *T. fuciformis*. In addition, differentially expressed genes from yeast and pseudohyphae will be screened in order to study the mechanism of dimorphic switching in *T. fuciformis*.

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