CHAPTER I

Introduction

Penicillium marneffei is the only temperature-dependent dimorphic fungus in the genus *Penicillium* (Cooper and Vanittanakom, 2008). This fungus grows as mycelia with hyaline septate hyphae and produces asexual conidia at the temperature below 37°C. When the temperature increases to 37°C as in human tissues or in culture medium, it forms yeast growth dividing by fission (Vanittanakom *et al*., 2006). This dimorphic switching is not found in other *Penicillium* species. The fungus causes a fatal systemic disease, penicilliosis marneffei, that has been found in southern China, Hong Kong, northeastern India and Taiwan and is endemic in Southeast Asia including Vietnam, Laos, Burma, Malaysia and Thailand (Cánvas and Andrianopoulos, 2007; Nelson *et al*., 2011). This disease is related to immunocompromised hosts especially in HIV patients and is known as an AIDS-indicator disease (Cooper and Vanittanakom, 2008). Infection is assumed to occur by inhalation of fungal conidia into the lungs similar to that of other dimorphic fungi, such as *Histoplasma capsulatum*. In the lungs, conidia are phagocytized by the alveolar macrophages of the host innate immune cells. In immunocompromised patients, conidia of *P. marneffei* can survive and kill the phagocytes resulting in the dissemination throughout host body (Cánovas and Andrianopoulos, 2007). For pathogenic fungi, if they are unable to overcome the host defensive mechanisms, especially reactive oxygen species (ROS) produced by host immune cells and other stresses inside the host microenvironment, they cannot establish the disease and will be eliminated from the host body (Arana *et al*., 2008). Therefore, to ensure survival inside macrophages and host tissues, the adaptation mechanism of the fungus is very important. This requires the fungus to sense and respond to host environmental stresses (Kummasook *et al*, 2007; Boyce *et al*., 2011). The adaptation mechanism of *P. marneffei* for survival against oxidative stress within host macrophages is still unknown. However, it has been shown that there were the

expression of acid phosphatase, the enzyme produced by some intracellular pathogens to inhibit the phagocyte respiratory burst, the Cu, Zn superoxide dismutase (Sod) and the high expression of catalase-peroxidase protein encoding gene (*cpeA*) at 37°C (Vanittanakom *et al*, 2006; Thirach *et al*., 2007; Pongpom *et al*., 2005). This indicates the ability of *P. marneffei* to respond to stresses for its survival inside human host cells.

The mitogen-activated protein kinase (MAPK) pathways are crucial signal transduction pathways found in several pathogenic fungi (Román *et al*., 2007). Inside the host body, the fungi have to succeed in adhering to host tissues, resisting host defense mechanisms and proliferating at certain sites to survive and cause diseases. The MAPK pathways are required for sensing and responding to the environmental signals that occur in the host including the change in pH, oxidative and osmotic stresses and nutrient starvation.

The members of MAPK family that are involved in stress-signaling molecules are stress-activated protein kinases (SAPKs). Like other MAPK pathways, each pathway of SAPKs is composed of three protein kinases: a MAPKK kinase (MAPKKK) or MEKK, a MAPK kinase (MAPKK) or MEK and a MAP kinase (MAPK). Under stress conditions, MEKK is activated and phosphorylates MEK. This results in the activation of MEK which then phosphorylates MAPK on both conserved serine and threonine residues in the catalytic domain. Phosphorylation of MAPK activates its kinase activity and transfer of this protein from cytosol to the nucleus. After activation, MAPK phosphorylates target proteins such as other kinases and transcription factors on serine/threonine followed by proline for triggering a suitable cellular stress response (Smith *et al*., 2010; Hohmann, 2002). The model of SAPK pathways includes the Hog1 pathway of yeasts *Saccharomyces cerevisiae* and *Candida albicans*, the Sty1 pathway of fission yeast *Schizosaccharomyces pombe*, and the SakA pathway of *Aspergillus nidulans*. Each pathway contains only one MAPK such as Hog1, CaHog1, Sty1, and SakA and one MAPKK including Pbs2, Wis1 and CaPbs2. Nevertheless, the number of MAPKKK proteins varies depending on the fungal species. This suggests that one SAPK pathway is able to response more than one stress signals. The Hog1 pathway plays an important role on osmotic adaptation of *S. cerevisiae*, whereas, the Sty1 pathway is involved in a global stress response in *S. pombe* including oxidative and

osmotic stress, UV light, heat shock and toxic cations (Hohmann, 2002; Vivancos *et al*., 2006). Interestingly, it has been shown that the MAPK pathways of some pathogenic fungi are involved in the control of virulence factors and pathogenesis including morphogenesis and stress adaptation (Román *et al*., 2007). For *A. nidulans*, SakA is required for resistance to hydrogen peroxide and heat shock stresses of germinated conidia (Kawasaki *et al*., 2002). In *C. albicans*, CaHog1 is essential for response to various stress conditions such as osmotic and oxidative stresses, antifungal drug and heavy metals treatments (Smith *et al*., 2010). *C. albicans hog1* mutant strains are more sensitive to phagocytic cells and less virulent than wild type strain in the systemic infection of mouse model (Román *et al*., 2007; Alonso-Monge *et al*., 1999). In addition, the *hog1* mutant cells are smaller and rounder than wild type cells and have a defect in yeast cell separation indicating the role of *hog1* in *C. albicans* morphogenesis (Xu, 2000; Alonso-Monge *et al*., 1999). The Hog1 pathway also plays a role in oxidative stress response and virulence of *Cryptococcus neoformans* (Román *et al*., 2007). *C. neoformans hog1* mutants are more sensitive to oxidative agents and the deletion of *hog1* in the serotype A, the most virulent strain, reveals attenuation of virulence in an animal model (Román *et al*., 2007; Bahn *et al*., 2005).

The systems that sense signals from the environments and transfer to the MAPK cascades are the two-component signaling systems. These systems are common signal transduction strategies found in both prokaryotes and eukaryotes using in response to environmental signals (Hagiwara *et al*., 2008). In fungi, these systems include multi-step phosphorelay proteins, a sensor histidine kinase protein (HK), a histidine-containing phosphotransfer (HPt) protein and a response regulator protein (RR) (Chauhan *et al*., 2006). In unstressed cells of *Saccharomyces cerevisiae*, there is an autophosphorylation on a histidine residue in a membrane-bound sensor kinase, Sln1. The phosphate is transferred to an aspartate residue on the receiver domain of the same protein and is subsequently transferred to a histidine residue in an HPt protein, Ypd1. Phosphorylated Ypd1 transfers phosphate to a response regulator, Skn7 or Ssk1 (Chauhan *et al*., 2006; Hohmann, 2002; Morgan et al., 1997). *S. cerevisiae* Skn7 is a response regulator that also acts as transcription factor and plays a role in antioxidation and cell-wall biosynthesis regulation (Chauhan *et al*., 2006). In pathogenic fungi, *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus*, Skn7 functions

in adaptation to oxidative stress and contribute to their virulence (Chauhan *et al*., 2006; Herrero *et al*., 2008). For Ssk1, under osmotic or oxidative stress, there is no phosphotransfer through Sln1-Ypd1-Ssk1 proteins. Unphosphorylated Ssk1 can activate the Hog1 MAPK pathway by binding to the MEKK protein, Ssk2. After activation, phosphorylated Hog1 translocates from cytosol to the nucleus and regulates transcriptions of genes involved in stress adaptation. In fission yeast *Schizosaccharomyces pombe*, under stress conditions, Sty1 translocates to the nucleus and phosphorylates the transcription factor Atf1 both *in vitro* and *in vivo*. Atf1, homolog of mammalian ATF2, is a basic-region leucine zipper (bZip)-type transcription factor that binds to the CRE sequence (T[G/T]ACGT[C/A]A) of the target genes in response to stress (Hagiwara *et al*., 2008; Sakamoto *et al*., 2009). In filamentous fungus, *Aspergillus nidulans*, stress activated kinase A, SakA (Hog1 homologue) translocates to the nucleus to interact with AtfA (Atf1 homologue) in response to oxidative or osmotic stress signal and AtfA also plays a role in oxidative and heat stress responses on conidia (Hagiwara *et al*., 2008; Lara-Rojas *et al*., 2010). For pathogenic dimorphic fungus *P. marneffei*, the homologues of genes encoding putative proteins in two-component signaling system including the histidine kinases (SlnA, DrkA) and the response regulator (Ssk1 and Skn7) and the putative proteins in the MAPK pathway such as the MAPKKK (Ssk2 and Ssk22) and the MAPK protein (SakA) have been identified in its genome (Boyce *et al*., 2011; Lin *et al*., 2012). Some of these putative proteins, their functions in stress response have also been investigated. it has been shown that the histidine sensor kinase SlnA and DrkA play a role in asexual development, hyphal morphogenesis, generation of yeast cell at 37ºC, cell wall integrity, osmotic adaptation and phosphorylation of SakA (Hog1) under osmotic stress (Boyce *et al*., 2011). In addition, the response regulator Skn7 encoding gene is involved in oxidative stress response (Cao *et al*., 2009). However, signal transductions under oxidative stress throughout the stress activated kinase (SAPK) pathway or SakA and AtfA transcription factor are not well understood.

The purpose of this study was to isolate *P. marneffei sakA* and *atfA* genes, generate the mutants deleted of these two genes and investigate their functions on oxidative stress response. *P. marneffei sakA* and *atfA* genes were amplified using specific primers and the nucleotide sequences were characterized by DNA sequence

analysis. The *P. marneffei sakA* and *atfA* mutant strains were generated using target gene deletion method. The phenotypes of the mutant strains including colony morphology, conidial production and susceptibility to oxidative stress were compared to the wild type strains. To confirm the functions of these two genes, the *P. marneffei sakA* and *atfA* complemented strains were constructed.