CHAPTER VI

Discussions

6.1 Isolation and characterization of *sakA* and *atfA* genes from *Penicillium marneffei*

Fungi are the examples of the organisms that have potential adaptation systems to wide ranges of environmental stress. Therefore, they are usually used as models to study the stress signaling pathways in eukaryotic cells. Stress-activated protein kinases (SAPKs) are members of the MAPK cascades that fungi use to transduce various forms of stress signals from the cell surface to the nucleus (Eaton *et al*., 2008; Wilkinson *et al*., 1996). These pathways are particularly conserved in eukaryotes and contain the similar basic mechanisms. The stress signals are transduced to the MAPKKK resulting in activation and phosphorylation of this protein. The phosphate is then sent to the MAPKK and subsequently sent to the threonine and tyrosine residue at the TXY phosphorylation lip of the MAP kinase protein (Hohmann, 2002; Ikner and Shiozaki, 2005).

In the present study, the results demonstrated that *P. marneffei sakA* gene encoded a putative protein containing the conserved TGY phosphorylation lip found in the stress Hog1/Spc1/p38 MAPK family at amino acid 171-173. This indicates that the *P. marneffei sakA* gene encodes a protein which is a member of the stress MAPK gene family. In addition, amino acid alignment of *P. marneffei* SakA revealed highly identical to filamentous fungi including *A. fumigatus* SakA (94%) and *A. nidulans* HogA (86%) indicating the close relationship between these two genera. Nevertheless, similar to *A. fumigatus* SakA, *P. marneffei* SakA does not contain the insertion of the highly acidic peptide (EEDED) presented in HogA of *A. nidulans* (Kawasaki *et al*., 2002).

After dual phosphorylation, activated MAP kinase usually phosphorylates and activates its target transcription factors (Román *et al*., 2007; Hohmann, 2002; Zhao *et*

al., 2007). The phosphorylation might occur in cytoplasm and the transcription factors translocate to the nucleus after activation or the MAP kinase translocates into the nucleus and the phosphate is then transferred to the transcription factors (Eaton *et al*., 1996). The activations of these transcription factors result in the expressions of the particular genes responsible for stress response. In mammalian cells, transcription factors regulated by MAPK pathways had been studied. The activating transcription factor 2 (ATF2) is found to be regulated by both JNK/SAPK and p38 MAPK cascades in response to stress stimuli or inflammatory cytokines (Tanoue and Nishida, 2003). The ATF2 homologues have been studied in both yeast and filamentous fungi. Atf1 of fission yeast *S. pombe* and AtfA of *A. nidulans* contain a bZip domain at their carboxyl terminus similar to mammanlian ATF2 and play a role in stress response (Hagiwara *et al*., 2008; Balázs *et al*., 2010; Vivancos *et al*., 2006; Papadakis *et al*., 2014).

The results in this study showed that *P. marneffei atfA* gene encoded a 409-amino-acid putative protein containing a conserved basic-leucine zipper (bZip) domain found in the bZip transcription factor family at the carboxyl terminus (amino acid 352-405) similar to Atf1 of fission yeast *S. pombe* and AtfA of *A. nidulans* . This suggests that *P. marneffei atfA* gene encodes a protein which is a member of the bZip-type transcription factor.

6.2 *sakA* and *atfA* gene expressions during phase transition and stress conditions

In the present study, it revealed that under normal condition, the expression of *P. marneffei sakA* gene in conidia was less than those in mycelial and yeast cells. However, under oxidative and heat stresses, the expression of this gene in conidia but not mycelia or yeast was increased. This suggests that *sakA* gene expression is activated in *P. marneffei* conidia under oxidative and heat stresses. In *A. nidulans*, the SakA MAP kinase is phosphorylated and activated under osmotic and oxidative stresses and *A. nidulans sakA* gene plays a role in conidial response against oxidative and heat stresses (Kawasaki *et al*., 2002).

On the other hand, *atfA* gene expression in *P. marneffei* conidia did not increased under both oxidative and heat stresses. This indicates that the expression of *atfA* gene is not induced under H_2O_2 stress and heat stress at 39ºC and the expression of this gene

might be activated by other oxidative stressors or other stress conditions. Similar to *A. nidulans*, *atfA* gene expression of this fungus is not be affected by H_2O_2, *t*-BOOH and NaCl stresses (Balázs *et al*., 2010).

6.3 Generations of *P. marneffei sakA* and *atfA* mutant strains using split marker method

The major problem for functional gene analysis in fungi is the high frequency of ectopic integration into their genomic DNA. The target gene deletion method is frequently used for functional analysis of genes of interest. Many genetic tools have been developed to increase the frequency of homologous recombination such as the deletion of genes involved in non-homologous recombination (*pkuA* or *ligD*) (Boyce *et al*., 2011; Bugeja *et al*., 2012) and the use of split marker method (Catlett *et al*., 2003). Eliminations of genes in non-homologous end joining highly increase the frequency of homologous recombination, however disruptions of some of these genes affect the DNA repair system of the recipient strains (Kück and Hoff, 2010). In split marker method, two DNA fragments containing flanking regions of target gene and partial sequence of marker gene are transferred to the wild type recipient. Three homologous recombinations of the flanking regions and the marker gene increase the frequency of homologous recombination at the desired position. Moreover, the split marker method does not require other genetic manipulation that will affect the genetic background of the recipient strain. However, the frequency of homologous recombination of this method depends on the recipient strains and the genes of interest and the transformation efficiency is lower comparing to the classical method (Kück and Hoff, 2010). In this study, to construct *P. marneffei sakA* mutant strain, the PCR-based system of the split marker method used for *A. fumigatus stuA* mutant construction was adapted (Sheppard *et al*., 2005). After transformation, only four transformants were presented on selective agar suggesting the low transformation efficiency. Nevertheless, one of these transformants (25%) revealed homologous recombination at the target gene.

For *atfA* mutant construction, the PCR-based method used in *sakA* gene was not successful. All of transformants grown on selective agar still contained *atfA* gene indicating the ectopic integration outside the target gene. This might depend on the nature of gene itself. However, the success of *atfA* mutant construction could be

obtained by using the modified split marker method with combination of plasmid-based and PCR-based systems (Catlett *et al*., 2003). Briefly, two DNA fragments were generated. One fragment was from the plasmid containing 5' flanking region and the first 500 bp of *atfA* gene fused with truncated sequence of *hph* gene. Another fragment containing 3'flanking region of *atfA* gene fused with truncated sequence of *hph* gene was the result from PCR amplification. After transformation of these two DNA fragments into *P. marneffei* wild type, there were five transformants presented on selective agar and one of them (20%) revealed homologous recombination at the target site.

6.4 Functional analysis of *P. marneffei sakA* and *atfA* genes

Responses to environmental stress are significant factors for many pathogenic fungi to survive outside and inside host cells and establish the disease. In this study, the results demonstrated that *P. marneffei sakA* played a role in conidia production, phase transition to yeast cell *in vitro* and survival inside macrophage cell lines, oxidative and heat stress tolerance, chitin deposition and cell wall integrity under cell wall stress at 37°C, whereas *P. marneffei atfA* participated in oxidative stress response and survival inside macrophage cell lines of the conidia.

Conidia production in *sakA* mutant strain was decreased when compared to those of wild type and *sakA* complemented strains suggesting the function of *sakA* gene in asexual development of *P. marneffei*. The defect in conidia production is also found in the *sakA* mutant of fungal endophyte, *Epichloë festucae*, *besak1* mutant of plant pathogen, *Botrytis cinerea* and *Bbhog1* mutant of entomopathogenic fungus, *Beauveria bassiana*. On the other hand, most stress-activated MAP kinase mutants of filamentous fungi reveal normal conidiation (Eaton *et al*., 2008; Segmüller *et al*., 2007; Zhang *et al*., 2009).

The ability in phase transition from the saprophytic mold phase in the environment to pathogenic yeast phase in the host cell is one type of dimorphism in fungi (Cooper and Vanittanakom, 2008). The transition of *P. marneffei* to yeast form within host macrophages is considered to be associated with fungal pathogenesis. However, pathways that regulate dimorphism in this fungus are still unclear. It has

been shown that phase transition in *P. marneffei* is determined by temperature and nutrition (Vanittanakom *et al*., 2006). In this study, conidia from all strains could transit to fission yeasts as found in clinical specimen when they were cultured in 1% peptone at 37°C. On the other hand, in BHI that is used to induce yeast cell generation *in vitro*, most conidia of *sakA* mutant strain were unable to transit to yeast form when compared to wild type and complemented strains. The same result was also shown in SDB medium suggesting that the *sakA* gene might be participated in phase transition to yeast form at 37°C in *P. marneffei*. The involvement of *hog1* gene in cell morphogenesis was also previously reported in *C. albicans* (Alonso-Monge *et al*., 1999). In the experiment of macrophage infection, the result showed that most of *sakA* mutant conidia were unable to germinate inside both THP1 and J774 cells and some of them germinated to hyphal cells. Moreover, survival of conidia from this strain inside macrophages was decreased suggesting the role of this gene in survival and yeast cell production inside macrophage. The same result was shown in *P. marneffei pakB* mutant. The *pakB* gene encodes a p21-activated kinase and is required for yeast cell production inside macrophage but not *in vitro* (Boyce *et al*., 2009). In addition, *P. marneffei* SlnA and DrkA encoding sensor histidine kinases are also involved in conidial germination and yeast cell transition inside macrophage (Boyce *et al*., 2011). These results indicate that *P. marneffei* utilizes more than one signaling pathway to regulate the transition to yeast cell inside host macrophages. For *atfA* gene, mutant of this gene did not affect the transition to yeast cell of conidia *in vitro*. Nevertheless, *P. marneffei atfA* mutant strain were more susceptible to both murine and human macrophages comparing to wild type and complemented strains indicating the involvement of *atfA* gene in survival of *P. marneffei* conidia inside macrophage cell lines similar to *sakA* gene. In *C. albicans*, *hog1* gene is responsible to oxidative-mediated killing by phagocytes. The *C. albicans hog1* mutant reveals decrease of survival inside human neutrophils (PMNs), HL-60 cells (promyelocytic cell line) and murine macrophages (Arana *et al*., 2007). Therefore, the results of this study suggest that *sakA* (*hog1* homologue) and *atfA* genes might participate in virulence of *P. marneffei*.

In *S. cerevisiae, hog1 (sakA* homologue) is involved in osmotic stress response (Zi *et al*., 2010). However, this study showed that conidia of *P. marneffei sakA* mutant

were similar to those of wild type strain in response to osmotic stress at both 25°C and 37°C. The same result was shown in *sakA* mutant of filamentous fungus, *A. nidulans* whose *sakA* mutant was indistinguishable from wild type strain under high osmolarity condition (Kawasaki *et al*., 2002; Lara-Rojas *et al*., 2011). These indicate that *sakA* gene is not involved in osmotic stress response in *P. marneffei* similar to those found in *A. nidulans*.

For cell wall integrity, *P. marneffei sakA* mutant has unusual chitin deposition along the hyphal cell wall, but growth of this strain is not different from the wild type and complemented strain under cell wall stress (CFW). This indicates that the abnormal chitin deposition at 25°C does not affect the cell wall integrity under cell wall stress. However, the mutant is slightly sensitive to CFW at 37°C and is more sensitive to SDS at both 25°C and 37°C. This suggests that the *sakA* gene seems to plays a minor role in cell wall integrity under cell wall stress. It has been shown that HOG pathway of yeast *S. cerevisiae* is activated and is required for cell wall integrity on cell wall stress response (Bermejo *et al*., 2008; Tomazett *et al*., 2011). In addition, the HOG pathway plays a role in cell wall biogenesis of *C. albicans* (Román *et al*., 2007). *C. albicans hog1* mutant has the defects in cell wall biogenesis and affects the transcription of histidine kinase, CHK1, which is required for oxidative adaptation, cell wall biosynthesis and virulence (Alonso-Monge *et al*., 1999; Kruppa and Calderone, 2006; Li *et al*., 2004).

For *atfA* gene, sensitivity of the conidia isolated from *P. marneffei atfA* mutant to osmotic stresses (NaCl and sorbitol) seemed to tolerate both NaCl and sorbitol better than the wild type and complemented strains. This might occur from compensation of the SakA pathway homolog. It has been shown that *A. nidulans* possesses two functional Hog1-type MAPKs including SakA/HogA and MpkC (Furukawa *et al*., 2005). Similar to HogA, MpkC can be phosphorylated by the MAKK protein (PbsB) and overexpression of *mpkC* gene can inhibit the high susceptibility to osmotic stress of *A. nidulans hogA* mutant (Furukawa *et al*., 2005; Miskei *et al*., 2009). In *A. fumigatus*, two Hog1 orthologues, SakA and MpkC participate in response to oxidative and nutritional stresses, respectively (Rispail *et al*., 2009). In addition, *A. fumigatus sakA* also shares a conserved role in osmotic stress response as in *S. cerevisiae* such that *A.*

fumigatus sakA controls the transcription of protein DprB required for osmotic and pH stress (Ma and Li, 2013). This indicates that overcompensation of *P. marneffei atfA* mutant strain to osmotic stress might come from the activation of the stress signaling pathway or transcription factor other than SakA-AtfA pathway. For cell wall stress, the susceptibility of conidia from the *atfA* mutant to CFW is similar to those of wild type and complemented strains. These results suggest that *P. marneffei atfA* might be involved in specific stress responses other than the cell wall stress responses. However, under stress from SDS, a membrane perturbation agent, survival of conidia of the mutant is less than those of the wild type and complemented strains at both 25ºC and 37ºC. This indicates the participation of this gene in cell membrane integrity.

Reactive oxygen species (ROS) produced by host immune cells such as macrophages, neutrophils and other phagocytic cells are toxic to some fungal pathogens and are able to eliminate these pathogens from the host body (Brown *et al*., 2009). Therefore, to protect themselves from host immunity, the stress response systems that can send the signal inside fungal cells to produce enzymes or molecules used to detoxify ROS are required. The results of this study showed that conidia of *P. marneffei sakA* mutant were more sensitive to both H_2O_2 and hydroperoxide (*t*-BOOH) than wild type and complemented strains while all strains revealed similar resistance to superoxide generator (menadione). In contrast to conidia, growth of mycelia from both wild type and *sakA* mutant under H_2O_2 and *t*-BOOH stresses were indistinguishable. This suggests that *sakA* gene plays a role in the oxidative stress response in *P. marneffei* conidia but not in mycelia.

For *atfA* gene, the results demonstrated that *P. marneffei atfA* gene was involved in response to only organic hydroperoxide, *t*-BOOH but not for H_2O_2 and menadione. These results indicated that *sakA* and *atfA* genes participated but did not play a major role in oxidative stress response in *P. marneffei*. In addition, *P. marneffei* might use different stress response systems or transcription factors to sense different ROS. In eukaryotic cells, different mechanisms used to defense against different oxidative compounds have been defined. Fungi utilize superoxide dismutases (SODs) to dismutate superoxide to hydrogen peroxide (H_2O_2) and molecular oxygen (O_2) (Gessler *et al*., 2007; Hamilton and Holdom, 1999). H_2O_2 is a byproduct of aerobic aspiration

and widely used as a model for oxidative stress generation. This molecule can be detoxified to H_2O and O_2 by catalase. *t*-BOOH is a simple organic alkylhydroperoxide that is frequently used to generate lipid oxidation. Glutathione peroxidase is used to reduce this toxic substance but not catalase (Morano *et al*, 2012; Kuge and Jones, 1994). It has been shown that conidia of *A. nidulans sakA* mutant are sensitive to menadione similar to those of wild type, but hypersensitive to both H_2O_2 and *t*-BOOH (Lara-Rojas et al., 2011). In yeast *S. cerevisiae*, only one polypeptide is induced under response to menadione whereas peroxide treatment leads to expression of at least 10 polypeptides (Flattery-O'Brien *et al*., 1993). In addition, cellular signaling response of this budding yeast activated by H_2O_2 is different from that is induced by *t*-BOOH (Morano *et al*, 2012; Kuge and Jones, 1994). Whereas Yap1, a bZip transcription factor of the AP-1 family plays a crucial role for tolerance to H_2O_2, diamide and cadmium in *S. cerevisiae*, the other transcription factor Cad1 is activated under *t*-BOOH treatment (Morano *et al*, 2012; Kuge and Jones, 1994) indicating that fungi use different antioxidant pathways in response to different oxidative compounds.

In *S. pombe*, Sty1 pathway is activated under diverse stress conditions including oxidative, osmotic stresses, UV light, and heat shock (Vivancos *et al*., 2006; Smith *et al*., 2010). In addition, conidia of *A. nidulans sakA* mutant are highly sensitive to oxidative and heat shock stresses (Kawasaki *et al*., 2002). In this study, *P. marneffei sakA* mutant was highly sensitive to heat shock at 39°C indicating the involvement of this gene in heat shock response. For *atfA* gene, deletion of *atfA* gene does not affect the susceptibility to heat stress at both 39ºC and 42ºC. Nevertheless, at 42ºC, the mRNA expression levels of both *sakA* and *atfA* genes in *P. marneffei* conidia were significantly increased. This suggests that heat shock stress might activate the expression of these two genes, but *atfA* gene does not play a major role in heat stress response in *P. marneffei*. In *S. cerevisiae*, it has been shown that there is cross protection among different stressors. Heat shock transcription factor (HSF1), MSN2 and MSN4 transcription factors play a major role in heat shock stress response and heat shock can stimulate tolerance to oxidative and osmotic stresses (Morano *et al*. 2012). In *A. nidulans*, AtfA plays a role in response against oxidative and heat stresses but not osmotic stress of conidia (Hagiwara *et al*. 2008). In *Aspergillus oryzae*, two genes encoding bZip type proteins similar to ATF/CREB, *atfA* and *atfB* have been reported

(Sakamoto *et al*., 2008). AtfB reveals a short N-terminal region comparing to AtfA and play a role in heat stress response and development of conidia under high osmotic stress. Nevertheless, *atfB* homolog in *P. marneffei* has not been reported.

For UV stress, *P. marneffei* wild type, *sakA* and *atfA* mutant conidia were exposed to different doses of UV light. The results showed that the survival of all strains after exposure to UV light were not significantly different suggesting that both *sakA* and *atfA* genes are not required for stress response against UV light.

Because the induction of the MAPK pathway results in the transcriptions of genes responding to environmental stresses, it is interesting to understand the relationship between the MAPK protein and the transcription factor inside the nucleus. In the present study, the functional analysis of *P. marneffei sakA* (*hog1* homologue) was performed and the results showed that this gene participated not only in heat stress response and oxidative tolerance to H_2O_2 and *t*-BOOH of the conidia but also involved in asexual development, yeast cell production at 37ºC, and chitin deposition along the hyphae. However, the outcomes from this study showed that *atfA* gene participated in a part of the systems regulated by *sakA* gene including tolerance to hydroperoxide (*t*-BOOH), SDS and survival inside macrophages. This indicates that SakA might interact with other MAPK proteins or other transcription factors to control gene expressions that are not dependent on AtfA. In *S. pombe*, the Sty1/Wis1 pathway is involved in osmotic, oxidative and heat stress responses and the control of mitotic initiation. It has been shown that *S. pombe* Atf1 directly binds and is phosphorylated by the Sty1 MAP kinase under these stress conditions. However, deletion of *atf1* did not have any effect on the timing of mitotic initiation (Wilkinson *et al*., 1996).

It has been shown that the regulation of ATF function is conserved. In mammalian, transcription factor ATF-2 is controlled by SAPK pathway similar to Atf1 of fission yeast *S. pombe* and AtfA of *A. nidulans* (Hagiwara *et al*., 2008; Wilkinson *et al*., 1996). The results from this study demonstrated that AtfA of *P. marneffei* might play a role downstream of SakA signaling pathway under certain stresses (SDS, *t*-BOOH and macrophage infection). However, further study on the interaction between SakA and AtfA under these stress conditions should be performed to help us understand more clearly the stress signaling pathways in dimorphic fungi. In addition, roles of these two

genes in virulence, pathogenesis and survival in host cells of *P. marneffei* are interesting and are remained to be discovered.