Communication

Molecular Cloning of Functional Genes for High Growth-Temperature and Salt Tolerance of the Basidiomycete Fomitopsis pinicola Isolated in a Mangrove Forest in Micronesia

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Several functional genes encoding putative proteins, heat shock protein 70, sphingosine phosphate lyase, and Na+/H+ antiporter, were cloned from the basidiomycete Fomitopsis pinicola, a wood-rotting fungus isolated in the tropical mangrove forest of Pohnpei Island of the Federated States of Micronesia. The deduced amino acid sequences of the obtained genes involved in heat shock resistance, lipid synthesis, and salt tolerance showed diverse similarities to other homologous proteins. Molecular phylogenetic trees of these proteins suggested that encoded proteins of the cloned genes of F. pinicola differed remarkably from other homologs in various organisms, even fungal proteins. Putative candidates for other genes related to several cellular metabolisms were also amplified, implying the possible existence of those genes in F. pinicola. This is the first report of possibly functional genes derived from a basidiomycetous mushroom growing in tropical islands such as Micronesia. The genes found in this study might play important roles in the cellular survival of the basidiomycete F. pinicola under severe environmental conditions.

Key words: basidiomycete; mangrove; Micronesia; tropical island; thermal and salt tolerance

In solitary Pacific islands including Pohnpei of Micronesia in the tropical area, the surface temperature over the Pacific Ocean is very high, and the daily change in atmospheric temperature at Pohnpei Island is more striking than in temperate regions. The humidity also changes drastically; the average reaches 90% and drops to 57.4% during a single day. Cell survival under severe thermal stress requires the activity of a chaperone system containing the HSP70 protein, a key member of ubiquitous molecular chaperones found in eubacteria and all branches of eukaryotes, including basidiomycetes. HSP70 chaperon plays central roles, possibly by providing an unfolding force that facilitates the extraction of misfolded proteins from aggregates. Lipids in cells and fatty acid composition are also known to play important roles in microbial temperature adaptation. For example, the filamentous fungus Neurospora, like basidiomycete, responds to changes in growth temperature by adjusting its lipid composition, and maintains either membrane fluidity or phase transition behavior. In Pohnpei Island, the salinity of air and ground water is generally higher than those on the continents. Na+/H+ antiporters are ubiquitous membrane proteins in most organisms, and are essential for adaptation to the high salinity of sea water. Here, to understand how the basidiomycete Fomitopsis pinicola isolated in a mangrove forest on Pohnpei Island adapts to high growth-temperatures and high salinity, we attempted to clone several functional genes involved in heat-shock resistance, salt tolerance, lipid synthesis, and also the rates of several metabolisms.

F. pinicola strain 130-2 derived from a basidiomycetous collection previously isolated in the forest on Pohnpei Island was grown in MYG medium (1% mol extract, 0.4% yeast extract, 1% glucose) at 30°C for several weeks with shaking. Fungal cells frozen in liquid N2 were disrupted with a homogenizer, and total DNA was extracted with an extraction buffer (0.1 M Tris–HCl, pH 8.0, 0.1 M EDTA, pH 8.0, 0.25 M NaCl, 0.1% N-lauroylsarcosine) after incubation at 55°C for 30 min. Amplification by polymerase-chain reaction (PCR) with approximately 60 ng of basidiomycete F. pinicola genomic DNA and 20 pmol of primers was carried out with 2.5 units of Taq polymerase (Toyobo Co., Ltd.) under slightly mild conditions (denaturing at 94°C for 30s, annealing at 42°C for 1 min, and extension at 70°C for 1 min, 32 cycles). The specific primer sets of the HSP70 gene, the sphingosine phosphate lyase gene, and Na+/H+ antiporter gene were originally designated by comparison of nucleotide

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Abbreviations: HSP70, heat shock protein 70; bp, base pair(s); kb, kilo base
sequences with encoded products of the corresponding genes in various organisms, which were retrieved from several databases: Genbank, EMBL, DDBJ, and SwissProt (Table 1). Amplified DNA fragments derived from the basidiomycete *F. pinicola* genome were electrophoresed on 1.5% agarose gel (Fig. 1). Some extra bands were observed in the cases of the HSP70 gene and the sphingosine phosphate lyase gene, and a single band of the Na+/H+ antiporter gene was detected (Fig. 1, lanes 1, 2, 3). Based on the predicted sizes of the amplified fragments, the corresponding bands, indicated by asterisks, were purified with a membrane-mediated spin column (Ultrafree-MC, AMICON, Millipore Corporation) and at least three identical clones of each gene were subjected to sequencing analyses on an ABI 310 fluorescent automatic DNA sequencer (PerkinElmer, Inc.). Computationally homological similarity searches of computational analyses of the databases, it was found that the three cloned genes encoded putative proteins: HSP70 protein, sphingosine phosphate lyase, and Na+/H+ antiporter protein respectively. Comparisons and alignments between the encoded amino acid sequences and those of homologs in various organisms are shown in Fig. 2. To clarify the extent of amino acid sequence similarities between the basidiomycete *F. pinicola* genes products and other homologs in more detail, phylogenetic trees of the various gene products were drawn (Fig. 3). Interestingly, none of *F. pinicola* gene products belonged to the branches of fungi *S. cerevisiae* and *Neurospora crassa* (Fig. 3A), *S. cerevisiae* (Fig. 3B), or also *S. cerevisiae, S. pombe*, or *N. crassa* (Fig. 3C).

In *Ustilago maydis*, a basidiomycetous fungus like *F. pinicola*, four genes of a HSP70-related gene family, *ums* 1–4, are transcriptionally active during normal growth. Following heat-shock treatment, the mRNA levels of *ums1* and *ums2* increase by approximately 5-fold, whereas the *ums3* transcript becomes less abundant and the amount of *ums4* mRNA remains relatively unchanged. A mutational analysis of *ums2* also suggested that *ums2* is essential for growth in *U. maydis*.

In such an equatorial region as Pohnpei island, the environmental temperature shift is often drastic, not only for 24 h but also between sunny places and sunshade regions, implying that heat-shock factors, including HSP70, are likely to play important roles in maintaining the homeostasis of cells in such situations. Interestingly, the basidiomycete *F. pinicola* 130-2 strain used in this work can grow optimally at 37 °C, although common basidiomycetes in the temperate regions grow at 25 °C, and cannot grow (or die) at 37 °C higher temperature than other temperate regions.

Table 1. Primer Sequences for Cloning of *F. pinicola* Genes by Polymerase Chain Reaction

<table>
<thead>
<tr>
<th>Amplified gene encoding product</th>
<th>Name</th>
<th>Length (bp)</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat-shock protein 70 (HSP70)</td>
<td>Hsp-F</td>
<td>20</td>
<td>5’-GATCTTCGAGGTCGCTGAAT-3’</td>
</tr>
<tr>
<td></td>
<td>Hsp-R</td>
<td>20</td>
<td>5’-CAGAGAATGCTGAGCATTGGT-3’</td>
</tr>
<tr>
<td>Sphingosine phosphate lyase</td>
<td>Spl-F</td>
<td>20</td>
<td>5’-TCCATCCGACGCTTCGATCGCT-3’</td>
</tr>
<tr>
<td></td>
<td>Spl-R</td>
<td>20</td>
<td>5’-TCTATCCGAATCCAGTGCTGTT-3’</td>
</tr>
<tr>
<td>Na+/H+ antiporter</td>
<td>Antiporter-F</td>
<td>24</td>
<td>5’-TTTCCCCGTGACGCTGACATTCCT-3’</td>
</tr>
<tr>
<td></td>
<td>Antiporter-R2</td>
<td>20</td>
<td>5’-GCCCACCAACCAAAACATGGA-3’</td>
</tr>
<tr>
<td>Pyruvate decarboxylase</td>
<td>PD-F</td>
<td>20</td>
<td>5’-CAACCCGACGCTGCTGTGTTT-3’</td>
</tr>
<tr>
<td></td>
<td>PD-R</td>
<td>20</td>
<td>5’-TCACCCCTCAACTTTGATGAGTG-3’</td>
</tr>
<tr>
<td>Elongation factor 1-α</td>
<td>EF1α-F</td>
<td>20</td>
<td>5’-GATCATCCAGTGTATGAGAT-3’</td>
</tr>
<tr>
<td></td>
<td>EF1α-R</td>
<td>20</td>
<td>5’-GTCGCAACATGATGGATGAGAG-3’</td>
</tr>
<tr>
<td>Actin</td>
<td>Act-F</td>
<td>20</td>
<td>5’-AACACCCACGGTGGAGGTT-3’</td>
</tr>
<tr>
<td></td>
<td>Act-R</td>
<td>20</td>
<td>5’-ATCGTTCGTGCTATGTT-3’</td>
</tr>
<tr>
<td>Alcohol dehydrogenase</td>
<td>ADH-F</td>
<td>20</td>
<td>5’-CGGAGAATGCTGGCTCAGGC-3’</td>
</tr>
<tr>
<td></td>
<td>ADH-R</td>
<td>20</td>
<td>5’-TGATGACGAGTATTTTCG-3’</td>
</tr>
<tr>
<td>Riboflavin aldehyde-forming enzyme</td>
<td>RFE-F</td>
<td>20</td>
<td>5’-GATGTCTCTGACCTGCTGAG-3’</td>
</tr>
<tr>
<td></td>
<td>RFE-R</td>
<td>20</td>
<td>5’-GATGTCTGAGCTTGTGATGTT-3’</td>
</tr>
<tr>
<td>Laminarinase</td>
<td>LAM-F</td>
<td>20</td>
<td>5’-GATCCAATGGAGCCGCGAGG-3’</td>
</tr>
<tr>
<td></td>
<td>LAM-R</td>
<td>20</td>
<td>5’-TACGGCGGCGGCAAGTACCAC-3’</td>
</tr>
</tbody>
</table>

In such an equatorial region as Pohnpei island, the environmental temperature shift is often drastic, not only for 24 h but also between sunny places and sunshade regions, implying that heat-shock factors, including HSP70, are likely to play important roles in maintaining the homeostasis of cells in such situations. Interestingly, the basidiomycete *F. pinicola* 130-2 strain used in this work can grow optimally at 37 °C, although common basidiomycetes in the temperate regions grow at 25 °C, and cannot grow (or die) at 37 °C. The amino acid sequence of the basidiomycete *F. pinicola* HSP70 gene product remarkably different even from fungal homologs (Fig. 2A, 3A) might be responsible for adaptation to such strict environmental conditions, especially to higher temperature than other temperate regions.
Fig. 1. Amplified Products Derived from the Basidiomycete *F. pinicola* Genome by Polymerase Chain Reaction.

Lane 1, the amplified product with primer sets of sphingosine phosphate lyase gene; lane 2, the amplified products with primer sets of HSP 70 gene; lane 3, the amplified product with primer sets of antiporter gene; M, molecular marker of 100-bp ladder. Arrowheads on the left sides of each panel indicate sizes of DNA fragments. Asterisks indicate the cloned fragments that were subjected to sequencing analyses. Experimental details are described in the text.

Fig. 2. Alignments of Amino Acid Sequences Encoded by the Obtained Genes of *F. pinicola* and Other Homologs in Various Organisms.

Numbers of amino acid residues of the corresponding protein are indicated on both sides. Identical amino acid residues are shown by asterisks above panels. Homologous amino acid residues are also shown by colons, and slightly similar residues are indicated by dots. Letters on the left indicate species as follows: Fp, *Fomitopsis pinicola*; Ec, *Escherichia coli*; Mm, *Mus musculus*; At, *Arabidopsis thaliana*; Sc, *Saccharomyces cerevisiae*; Nc, *Neurospora crassa*; Dm, *Drosophila melanogaster*; Hs, *Homo sapiens*; Ce, *Caenorhabditis elegans*; Sp, *Schizosaccharomyces pombe*; Dd, *Dictyostelium discoideum*. Potentially predicted regions (extracellular, *Ex*, transmembrane, *Tr*, and cytoplasmic, *Cy*) are indicated under the alignment.
Sphingolipids elicit a wide variety of eukaryotic cellular responses, most involving regulation of cell growth, differentiation, and apoptosis.\(^{16}\) Sphingosine-1-phosphate, a sphingolipid, has been found to participate in the proliferative signal transduction pathways of cells,\(^{17}\) and its degradation requires cleavage at the carbon bond by sphingosine phosphate lyase.\(^{16}\) At the present time, only few works on sphingosine phosphate lyase have been reported\(^{18}\) (refer to Fig. 2B, 3B), and the first identification of the mammalian sphingosine phosphate lyase gene was described in 1998.\(^{17}\) A recent work on nematode *Caenorhabditis elegans* suggested that sphingosine phosphate lyase is an essential gene in *C. elegans*, and that the sphingolipid degradative pathway plays a conserved role in regulating cellular development.\(^{18}\) In previous study of the mutual interactions *in vitro* between glycoproteins and sphingolipids within the cellular membrane, the thermotropic behavior of the glycoprotein showed that the temperature of irreversible thermal unfolding of the glycoprotein, centered on 65.9 °C in the absence of sphingolipids, shifted to 57.6 °C when sphigolipids were present in the bilayer, suggesting that sphingolipid clusters affect protein conformation and oligomerization in the membrane under thermal environmental condition.\(^{19}\) Experimental results on bovine oocytes have demonstrated that Sphingosine-1-phosphate protected oocytes from physiologically relevant heat-shock and then the survived oocytes normally differentiated to blastocysts.\(^{20}\) In *N. crassa*, a filamentous fungus like *F. pinicola*, plasma membranes isolated from a mutant of *N. crassa* grown at 37 °C and at 15 °C displayed large differences in lipid compositions of sphingolipid and phospholipid species, suggesting that coordinate modulation of those lipid compositions may be involved in the regulation of plasma-membrane fluid properties during temperature acclimation.\(^{21}\) Moreover, the total amount of phospholipids remarkably increases in fruiting bodies of the basidiomycete *Lentinula edodes*, and the compositions of fatty acyl residues play important roles in cell growth and differentiation, especially in temperature shift.\(^{22}\) An analysis of developmentally regulated genes in *L. edodes* suggested that specific molecular mechanisms surrounding cellular lipids occurred during fruiting body development.\(^{23}\) These data strongly imply the existence of a relationship between lipid synthesis and the growth temperature of basidiomycete. Thus, the *F. pinicola* sphingosine phosphate lyase gene obtained in this work may also play an important role in the regulation of lipid composition to adapt to the high temperature of the tropical climate in Micronesia, also in view of its amino acid sequence considerably different from *S. cerevisiae*, a fungus like *F. pinicola* (Fig. 2B, 3B).

In the budding yeast *Saccharomyces cerevisiae*, a eukaryotic fungus like basidiomycete, the Na\(^+\)/H\(^+\) antiporter has been reported to play an important role in maintaining intracellular pH and Na\(^+\) homeostasis.\(^{24}\) Overexpression of an Na\(^+\)/H\(^+\) antiporter by genetic engineering conferred salt tolerance on a freshwater cyanobacterium and made it capable of growth in sea water,\(^{25}\) whereas a variety of filamentous fungi has recently been isolated from diverse places including the Dead Sea, which contains 340 g/litter total dissolved salts.\(^{26}\) The vegetation in Pohnpei Island are extremely salt-tolerant and show no adverse salinity effects,\(^{2}\) and so basidiomycetous fungi grown there are likely to possess a high tolerance for salinity. Indeed, most strains of *F. pinicola* isolated in Pohnpei Island can grow in a medium containing over 0.8 M NaCl (approximately 47 g/litter),\(^{10}\) implying that particular mechanisms such as an antiporter system may play crucial roles in maintaining ion homeostasis in fungal cells. The differences in the amino acid sequence encoded by the putative antiporter gene of *F. pinicola* in this work may be related to higher salt-tolerance than that of basidiomycetes commonly found in the temperate regions.

To examine further whether other functional genes also exist in *F. pinicola*, we carried out more PCR analyses. Specific primers of several genes encoding pyruvate decarboxylase, elongation factor 1-a, actin, alcohol dehydrogenase, riboflavin aldehyde-forming enzyme, and laminarinase (Table 1) were designed according to our previous data on fruiting-specific genes in the basidiomycete *L. edodes*.\(^{23}\) Several amplified DNA fragments were detected in each case (data not shown). The possibility that those products contain the corresponding genes, however, remains to be examined.

In comparison with other homologs in several
organisms, the remarkable differences in amino acid sequences of the three cloned genes (see Fig. 2, 3) may be caused by an original and a unique evolution of *F. pinicola*, because Pohnpei is a solitary island extremely far off in the sea. Further analyses of various kinds of organisms in isolated regions such as the Micronesian Islands are likely to reveal evolutionary differences in gene structures and encoded proteins and to provide a novel point of view of biological interest. Finally, the findings presented here support the conclusions that the obtained genes may be related to several cellular mechanisms, including a heat-shock protein system including HSP70, a salt tolerance system, and the regulation of lipid composition for high temperature and high salinity in the basidiomycete *F. pinicola*. The proteins encoded by functional genes in this work play possible roles for survival on Pohnpei island, nearly on the equator, and Micronesian microorganisms including basidiomycetous fungi such as *P. pinicola* may have “special” proteins and mechanisms to resist such the severe environmental conditions unique to tropical regions and/or islands.

Acknowledgment

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References

bacterium, making it capable of growth in sea water. 


26) Kis-Papo, T., Oren, A., Wasser, S. P., and Nevo, E., 
Survival of filamentous fungi in hypersaline Dead Sea 

27) Domanico, S. Z., DeNagel, D. C., Dahlseid, J. N., Green, 
J. M., and Pierce, S. K., Cloning of the gene encoding 
peptide-binding protein 74 shows that it is a new 
member of the heat shock protein 70 family. Mol. Cell. 

28) Kim, H., Melen, K., Osterberg, M., and von Heijne, G., 
A global topology map of the Saccharomyces cerevisiae 
membrane proteome. Proc. Natl. Acad. Sci. USA, 103, 