농학석사학위논문

Functional analysis of genes encoding membrane-bound transcription factor in the rice blast fungus, *Magnaporthe oryzae*

벼 도열병균 막결합전사인자 유전자들의 기능 규명

2013년 8월

서울대학교 대학원

농생명공학부 식물미생물학전공

정 수 빈

A THESIS FOR THE DEGREE OF MASTER OF SCIENCE

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BY

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위 원 

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UNDER THE DIRECTION OF DR. YONG-HWAN LEE

SUBMITTED TO THE FACULTY OF THE GRADUATE
SCHOOL OF SEOUL NATIONAL UNIVERSITY
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SUBIN JUNG

MAJOR IN PLANT MICROBIOLOGY
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BY THE COMMITTEE MEMBERS

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Functional analysis of genes encoding membrane-bound transcription factor in the rice blast fungus, *Magnaporthe oryzae*

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In Agricultural Biotechnology

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ABSTRACT

Membrane-bound transcription factors (MTFs) are known as key regulators which response to internal and external changes for homeostasis and adaptation, respectively. MTFs bind to membrane by transmembrane motifs as a dormant form, but DNA binding motifs are cleaved from membrane and move directly to nucleus by specific stimuli. In plants and animals, several MTFs have been characterized to have a role in sterol homeostasis and stress response. In fungi, however, the functions of MTFs are largely unknown in aspect of stress response and phytopathogenic activity. Objectives of this study are 1) identification of genes encoding MTFs in the genome of the phytopathogenic

fungus *Magnaporthe oryzae*, and 2) functional analysis of MTF genes by targeted gene knockout strategy. A total of 481 TF genes predicted from FTFD (Fungal Transcription Factor Database: http://ftfd.snu.ac.kr) were used to identify MTFs by using TMHMM 2.0 program. Initially 14 MTF gene candidates were identified, but 6 genes of them encoded putative palmitoyltransferases. By manual curation, one ortholog of Sre1 gene was added, resulting in 9 putative MTF genes in *M. oryzae*. Comparative and phylogenetic analyses were conducted to understand their ortholog relationships. Among 9 putative MTFs, 5 knockout mutants, **Δ***Momtf2, 4, 5, 6, 8* were generated. Mutants were indistinguishable to wild-type in mycological characteristics growing in normal condition and pathogenic activity. Higher expression of MTF genes in osmotic-, ionic-, oxidative-, temperature-stress, and starvation conditions suggested their roles in response to external changes. Observation of mycelial growth under stress conditions also supported that MoMTFs play roles in stresses adaptation. GFP-tagged MoMTF5 protein was located in both of plasma-membrane and nuclei. These results support that *MoMTFs* in *M. oryzae* are required for environmental fitness.

KEYWORDS: *Magnaporthe oryzae*, Membrane-bound transcription factor gene, Transmembrane motif, Targeted knockout, Stress adaptation

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INTRODUCTION

The rice blast occurred through leaves to grains in entire lifecycle of rice plant and caused serious crop loss worldwide. Causal agent is ascomycete filamentous fungus, *Magnaporthe oryzae* which is important model organism for understanding of molecular host-parasite interaction (Valent, 1990). Full genome sequence of both rice (Goff et al., 2002; Yu et al., 2002) and *M. oryzae* (Dean et al., 2005) had been released and a total of 12,991 genes were annotated in *M. oryzae* genome.

Transcription factors (TFs) have a crucial role as transcriptional key regulators, so they have DNA binding domain to initiate or repress target genes. TFs are classified into different family according to their different type of DNA binding domain. In *M. oryzae*, at least 481 (3.7% of total annotated genes) putative TF genes were reported in previous study (Park et al., 2008a).

Membrane-bound transcription factors (MTFs) are specific type of TFs that anchor in intracellular- or plasma-membrane as a dormant form. When they are stimulated, it cleaves from membrane and be an activate form. This is a distinguishable character, so that expecting that MTFs function dissimilarly with other TFs. Previous study said, MTF's activation is induced

by various stresses and environmental changes. In animal, the SREBP (steroid response element binding protein) TF is controlled by intracellular cholesterol level for sterol homeostasis (Hua et al., 1993; Wang et al., 1994; Eberle et al., 2004). Activation of MTFs in plant is related with diverse abiotic stresses such as ER stress or high salt (Iwata and Koizumi, 2005; Liu et al., 2007; Chen et al., 2008; Liu et al., 2008; Tajima et al., 2008; Kim et al., 2010). In fungi, only the MTFs showing homology with animal SREBP were characterized. They function in ergosterol (a kind of sterol found in fungal species) synthesis similar with SREBP proteins and also adaptation to hypoxia circumstance (Hughes et al., 2005; Chang et al., 2007; Willger et al., 2008; Bien and Espenshade, 2010). Interestingly, Fungal MTFs are also related to virulence in pathogenic- and opportunistic fungi, such as *Cryptococcus neoformans, Aspergillus fumigatus and Candida albicans* (Espenshade and Hughes, 2007). In baker's yeast *Saccharomyces cerevisiae*, there is no SREBP-homolog proteins but have MTFs belonging to NF-κB like TF family. They function in unsaturated fatty acid homeostasis (Hoppe et al., 2000; Hitchcock et al., 2001).

Until now, almost researches of fungal MTFs were focused in SREBP homologs unlike studies in plant and animal. Moreover, MTFs in phytopathogenic fungi, including *M. oryzae* are not characterized yet. For

these reasons, study about MTFs in rice blast fungus, *M. oryzae* is needed. In this study, it was focused to identify the function of MTFs particularly about environmental change/stress adaptation.

Table 1. Previous studies about membrane-bound transcription factor

Gene name	TF family	Species	Localization	Activated by	Processing	Function	References
SREBP-1 (*SREBP-1a* ,*SREBP-1c*)	bHLH	*Homo sapiens*	Nuclear envelope / ER	Sterol depletion (SREBP-1a) Insulin (SREBP-1c)	RIP	Cholesterol synthesis, Fatty acid synthesis, Insulin-induced glucose metabolism	(Wang et al., 1994; Eberle et al., 2004)
SERBP-2	bHLH	*Homo sapiens*	ER	Sterol depletion	RIP	Cholesterol synthesis	(Hua et al., 1993; Eberle et al., 2004)
dSREBP	bHLH	*Drosophila Melanogaster*	ER	Palmitic acid, Phosphatidyl-ethanolamine	RIP	fatty acid and phospholipid synthesis	(Dobrosotskaya et al., 2002; Seegmiller et al., 2002; Matthews et al., 2009)
SBP-1	bHLH	*Caenorhabditis elegans*	Unknown	Starvation, hypoxia	Unknown	Fatty acid synthesis, Regulate amount / composition of fat	(Taghibiglou et al., 2009; Nomura et al., 2010)
ATF6	bZIP	*Homo sapiens*	ER	ER stress	RIP	Unfolded protein response	(Haze et al., 1999)
OASIS	bZIP	*Rattus norvegicus*	ER	ER stress	RIP	Unfolded protein response	(Murakami et al., 2006)

Table 1. (continued)

Gene name	TF family	Species	Localization	Activated by	Processing	Function	References
AtbZIP60	bZIP	*Arabidopsis thaliana*	ER	ER stress	RIP	Unfolded protein response	(Iwata and Koizumi, 2005; Chen et al., 2008)
AtbZIP28	bZIP	*Arabidopsis thaliana*	ER	ER stress	RIP	Unfolded protein response	(Liu et al., 2007; Tajima et al., 2008)
AtbZIP17	bZIP	*Arabidopsis thaliana*	ER	Salt stress	RIP	Salt stress response	(Chen et al., 2008; Liu et al., 2008)
NTM1	NAC	*Arabidopsis thaliana*	Nuclear envelope / ER	Cytokinins	RIP	Cell cycle mediates cytokinin signaling	(Kim et al., 2006)
NTL8	NAC	*Arabidopsis thaliana*	plasma membrane	Salt stress	RIP	Salt regulation of seed germination	(Chen et al., 2008)
NTL9	NAC	*Arabidopsis thaliana*	plasma membrane	Osmotic stress	Unknown	Osmotic stress response Leaf senescence	(Chen et al., 2008)

Table 1. (continued)

Gene name	TF family	Species	Localization	Activated by	Processing	Function	References
Sre1	bHLH	*Schizosaccharo-myces pombe*	Unknown	Sterol depletion, hypoxia	RIP	Ergosterol synthesis Adoptation to hypoxia	(Hughes et al., 2005; Bien and Espenshade, 2010)
Sre2	bHLH	*Schizosaccharo-myces pombe*	Unknown	Constitutively activated	Unknown	Unknown	(Hughes et al., 2005; Bien and Espenshade, 2010)
SRE1 (*CnSre1*)	bHLH	*Cryptococcus neoformans*	Unknown	Sterol depletion, hypoxia	RIP	Ergosterol synthesis Adoptation to hypoxia Iron and copper uptake Virulence	(Chang et al., 2007; Bien and Espenshade, 2010)
SrbA	bHLH	*Aspergillus fumigatus*	Unknown	Sterol depletion (indirect), hypoxia	Unknown	Ergosterol synthesis Adoptation to hypoxia Cell polarity and cell wall maintenance Virulence	(Willger et al., 2008; Bien and Espenshade, 2010)
Cph2	bHLH	*Candida albicans*	Unknown	Unknown	Unknown	Filamentous growth Virulence	(Lane et al., 2001; Bien and Espenshade, 2010)
SPT23	NF-κB like	*Saccharomyces cerevisiae*	nuclear envelope / ER	Fatty acid pools	RUP	Control unsaturated fatty acid levels	(Hoppe et al., 2000; Hitchcock et al., 2001)
MGA2	NF-κB like	*Saccharomyces cerevisiae*	nuclear envelope / ER	Unknown	RUP	Control unsaturated fatty acid levels	(Hoppe et al., 2000; Hitchcock et al., 2001)

MATERIALS AND METHODS

I. Fungal strains and culture conditions

Magnaporthe oryzae strain KJ201 was provided by the Center for Fungal Genetic Resources (CFGR, http://genebank.snu.ac.kr) and used as the wild-type strain. Wild-type and its knockout mutant strains were maintained on solid V8 juice media (8% V8 vegetable juice (v/v) and 1.5% agar powder) and solid oatmeal media (5% oatmeal (w/v) and 2.5% agar powder) at 25℃ under continuous fluorescent light. For genomic DNA extraction, wild-type and mutant strains were cultured in liquid complete medium (0.6% yeast extract (w/v), 0.6% casamino acids (w/v), and 1% sucrose (w/v)) at 25℃ in dark condition for 5 days.

II. Identification of putative membrane-bound transcription factors

List of putative transcription factors in *M. oryzae* was obtained from Fungal Transcription Factor Database (FTFD; http://ftfd.snu.ac.kr) (Park et al., 2008a) and Comparative Fungal Genetics Platform (CFGP; http://cfgp.snu.ac.kr) (Park et al., 2008b; Choi et al., 2013). All gene sequences were saved as a FASTA format and analyzed by TMHMM 2.0 server (http://www.cbs.dtu.dk/services/TMHMM) (Krogh et al., 2001) to

predict transmembrane motifs. To search homolog and clarify the homolog relationships, BLAST in National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov) was used. Protein sequences were aligned and phylogenetic trees were constructed by Neighbor-joining method (Saitou and Nei, 1987) with bootstrapping (1,000 replicates) using MEGA 5.0 program (Tamura et al., 2011).

III. Nucleic acid manipulation

Genomic DNA was prepared two different extraction methods under their experimental purposes. Multi-purpose genomic DNA, such as targeted knockout PCR, fungal transformation, enzyme digestion and Southern hybridization was made standard genomic DNA extraction method. For PCR screening experiment, direct PCR method was performed. DNA probes for Southern hybridization were labeled with P^{32} by Rediprime^{TM} II Random Prime Labeling System Kit (GE healthcare, NJ, USA). In order that hybridized membrane was visualized, it was exposed on BAS-2040 image plate (Fuji Photo Film, Tokyo, Japan) and red by Phosphorimager software. Total RNA for expression analysis was isolated by using the Easy-spin^{TM} total RNA extraction kit (Intron Biotechnology, Seoul, Korea).

IV. Disruption of putative membrane-bound transcription factors in *M. oryzae*

Double-joint PCR (Yu et al., 2004) was performed with extracted genomic DNA of *M. oryzae* strain KJ201 for knockout DNA construct. The location of target gene was substituted *HPH* marker cassette amplified from the plasmid possessing hygromycin B phosphotransferase (pBCATPH vector) (Kim et al., 2005) using three primer pairs, 5' Forward/5' Reverse, HygB_F/HygB_R, and 3' Forward/3' Reverse for antibiotic resistance (Table 1).

Protoplasts of *M. oryzae* strain KJ201 were transformed by PEG-mediated fungal transformation (Klebe et al., 1983). After transformation, mutant candidates were screened by two times of PCR-based screening procedures and purified by single spore isolation. Direct PCR method in which small piece of mycelium was used as PCR template without DNA extraction.
Finally, mutants were confirmed by Sothern blot analysis and used for this study.

V. Characterization of MoMTF deletion mutants

Mycelia growth was measured with tree replacements on Talbot modified complete agar medium (CM) (Talbot et al., 1993) on 9 days after inoculation.

All of used inoculums were generated from minimal media (MM) over 6-day-old. The number of conidia was counted for measuring of the ability of asexual reproduction. Conidia harvested from 6-day-old V8 juice agar media by resuspending with 5 mL of sterile distilled water. 20 μL of collected conidial suspension observed on the hemocytometer. To assess conidial germination and appressorium formation, conidial suspension of 40 μL was dropped on the hydrophobic coverslip with three replicates. At this time concentration of conidial suspension was adjusted to 2×10^4 spores/mL and incubated at 25℃ in moist condition. After 4 hours and 24 hours, it was observed the range of germinating and germinated conidia-forming appressoria by microscope with least 100 conidia and three replicates each experiment, respectively.

For virulence test, conidia harvested from 6- to 10-day-old V8 juice agar culture. Adjusted conidial suspension (5×10^4 spores/mL) by sterile distilled water containing 250 ppm Tween 20 was sprayed to 3- to 4-week old rice seedling (*Oryza sativa* cv. Nakdongbyeo). Infected rice was kept in dew chamber (25℃, 100% humidity, continuous dark condition) for 24 hours and moved to growth chamber (25℃, 60% humidity, 16-h-light/8-h-dark cycle) for disease development.

To evaluate the stress susceptibility, vegetative growth of wild type and

mutants strains was observed under conditions as follows; nitrogen starvation, carbon starvation, stress condition for cell wall biogenesis (200 ppm calcofluor white in solid CM), stress condition for protein synthesis (1 mM cycloheximide in solid CM), oxidative stress conditions (2.5 mM methyl viologen or 5 mM hydrogen peroxide in solid CM), detergent condition (0.01% SDS in solid CM), and ionic stress conditions (200 mM calcium chloride or 20 mM manganese (II) chloride in solid CM).

To observe availability of non-fermentable carbon source, wild type and mutants were grown on solid carbon starvation media supplemented with 2% lactate, 2% glycerol, or 2% sucrose.

VI. Quantitative RT-PCR and gene expression analysis

To analyze expression level between KJ201 to each mutant, quantitative RT-PCR was used. To extract RNA under stress and starvation conditions, *M. oryzae* wild type strain KJ201 were treated as follows; complete medium (fully fed, used as control), 1 M mannitol, 1 M sorbitol, 1 M potassium chloride, 0.4 M sodium chloride, 1 M sodium chloride, 1 M lithium chloride, 50℃ (heat shock), 0℃ (cold shock), 2.5 mM hydrogen peroxide, 5 mM methyl viologen, nitrogen-starvation, and carbon-starvation. Messenger RNA in each 5 μg of total RNA sample was reverse transcribed into cDNA using

oligo(dT) primer and ImProm-II Reverse Transcription System kit (Promega, Madison, WI, USA) following a manufacturer's instruction. For reaction, primer pair designed to amplify a small piece of DNA in exon region, cDNA, SYBR Green PCR Master Mix (2X) (Applied Biosystems) were mixted to make 10 μL volume and analyzed by ABI7500 Real-Time PCR system (Applied Biosystems).

VII. Cellular Localization of MoMTF5 in *M. oryzae*

To identify subcellular location of putative membrane-bound transcription factor proteins in *M. oryzae*, MoMTF5 was tagged with green fluorescent protein (EGFP). Double joint PCR (Yu et al., 2004) joined three DNA fragments, *Aspergillus nidulans* TrpC promoter, EGFP gene, and MoMTF5 gene with native terminator to generate TrpC-EGFP-MoMTF5 construct. TrpC-EGFP-MoMTF5 and geneticin resistance marker (pII99 vector) (Lee et al., 2003) were introduced to protoplast of *M. oryzae* strain KJ201 by PEG-mediated fungal co-transformation. Transformants survived in regeneration media (TB3; 1% glucose (w/v), 20% sucrose (w/v), 0.3% yeast extract (w/v), 0.3% casamino acids (w/v), and 0.8% agar powder) containing geneticin (200 ppm G-418) were screened by PCR-based screening. Positive transformants were grown on V8 juice agar media to promote conidiation

and fluorescence signals in conidia were observed under fluorescence microscope (Zeiss Axio Imager A1; Cal Zeiss) with GFP filter.

For nuclear staining of transformants, Hoechst33342 was added to conidial suspension and washed several times with sterile distilled water after 10 minutes incubation. Fluorescence signals were observed under fluorescence microscope (Zeiss Axio Imager A1; Cal Zeiss) with 4',6-diamidino-2-phenylindole filter.

Table 2. Primer list used in this study

Name	Sequence (5'→3')
Hyg B-f	CGACAGAAGATGATATTGAAGG
Hyg B-r	CTCTAAACAAGTGTACCTGTGC
HPH_Sr2	GGCTGATCTGACCAGTTGC
HPH_Sf4	CAAGCCTACAGGACACACATTC
MoMTF2_5F	CAGCATAGCAACACAGGATGGATC
MoMTF2_5R	GCACAGGTACACTTGTTTAGAGAGCTGTCGGGACAAGTAGTTGGAC
MoMTF2_3F	CCTTCAATATCATCTTCTGTCGACTTGACCTGGAAGATGGGCTAAGC
MoMTF2_3R	GCTCGAGACCCCAATACAAGCG
MoMTF2_Sf	CAACACAGGATGGATCAGAG
MoMTF4_5F	GCCGATGCTGCGACTAACGA
MoMTF4_5R	GCACAGGTACACTTGTTTAGAGAGCGTGCTTGAGCTGATGACC
MoMTF4_3F	CCTTCAATATCATCTTCTGTCGAACTTCTCGCCTCGGTTTCTGCTA
MoMTF4_3R	CAAACTGGGGTGATGTCGCAAT
MoMTF4_Sf	TGGCAGCGTATTTATCGTG
MoMTF5_5F	CCTGACGAGCCTATTACCTG
MoMTF5_5R	GCACAGGTACACTTGTTTAGAGACCACTTGGGTCTAGCACTACAG
MoMTF5_3F	CCTTCAATATCATCTTCTGTCGAGGGTCTAGGTCTGTAAAACGAG
MoMTF5_3R	CGGCGATGTATCAGCAAAC
MoMTF5_Sr	CCCTGTCGGAAGTCCTTAC
MoMTF6_5F	GAGGTCCACCCATAATCAGTTC
MoMTF6_5R	GCACAGGTACACTTGTTTAGAGAGACCAAACAGGATAGGCAAAAG
MoMTF6_3F	CCTTCAATATCATCTTCTGTCGATACAGAAACGGAGGACATACC
MoMTF6_3R	GGCTACGAAAACCTCACTACTG
MoMTF6_Sr	GCGTCCTAGATCGTTATTACCC
MoMTF8_5F	CCGTGTAATACCGACTTCCGTCG
MoMTF8_5R	GCACAGGTACACTTGTTTAGAGAGTTCAACCGCAACTTGCTACGC
MoMTF8_3F	CCTTCAATATCATCTTCTGTCGAGGCATTTCTTGCCATCCTCTGTAG
MoMTF8_3R	GTAGTAAACGGCGCCATGGC
MoMTF8_Sf	AGCGTGGCTATGTTGTACC
Ef1α_F	CGGTACCTATAGGGCGAATTG
Ef1α_R	CTCGCCCTTGCTCACCATTTTGAAGATTGGGTTCCTTTTG
EGFP_F	ATGGTGAGCAAGGGCGAG
EGFP_R	CTTGTACAGCTCGTCCATGC
pTrpC_F	GTCGACAGAAGATGATATTGAAG
pTrpC_R	CTCGCCCTTGCTCACCATCATATCGATGCTTCGGTAGA
MoMTF5_EGFP_F	GCATGGACGAGCTGTACAAGATGGAGATTGACAGTCGCC
MoMTF5_EGFP_R	GATGGCAGGCTCTCAATCTC

RESULTS

I. Identification of genes encoding putative membrane-bound transcription factors

According to FTFD, a total of 481 genes were assigned to be putative TFs in *M. oryzae* (Park et al., 2008a) and TMHMM 2.0 server identified 14 candidates of membrane-bound TFs with putative transmembrane motifs. BLAST analysis, then revealed homolog proteins of 14 candidates and 6 of them were filtered out because of their homolog relationship with palmitoyltransferases. The excluded 6 candidates belonging to Zinc-finger DHHC type protein family may play a role in post-transcriptional regulation. In addition to the rest of 8 members of putative membrane-bound TF, one locus (MGG_11534) displaying homology with SREBP (sterol regulatory element binding protein) and Sre1 in animal and fungi, respectively, was also included. As a result, 9 putative membrane-bound TFs were identified in *M. oryzae* and named as MoMTF1-9 (Table 3). Gene structure analysis showed that there are 4 proteins of Zn_2Cys_6 TF family, 2 proteins of nucleic acid-binding OB-fold family, and 3 proteins each of them belongs to helix-turn-helix AraC type, HMG-I DNA-binding, basic helix-turn-helix family. The

numbers of transmembrane motifs in protein sequences ranged from 1 to 11. More than half of MoMTFs have over 2 transmembrane motifs (Fig. 1).

Table 3. List of putative membrane-bound transcription factor in *M. oryzae*

TF family	Gene name	Locus	Size (aa)	TM Count	Homologues
Helix-turn-helix, AraC type	*MoMTF1*	MGG_08970.7	1369	11	Leptomycin B resistance protein pmd1
HMG	*MoMTF2*	MGG_05185.7	562	2	-
Nucleic acid-binding, OB-fold	*MoMTF3*	MGG_04335.7	685	6	-
	MoMTF4	MGG_08600.7	270	5	-
Zn2Cys6	*MoMTF5*	MGG_02880.7	827	1	Transcriptional activator xlnR
	MoMTF6	MGG_05683.7	973	2	-
	MoMTF7	MGG_05829.7	656	1	-
	MoMTF8	MGG_09027.7	770	1	-
bHLH	*MoMTF9*	MGG_11534.7	1028	(2)*	Regulator of sterol biosynthesis Sre1

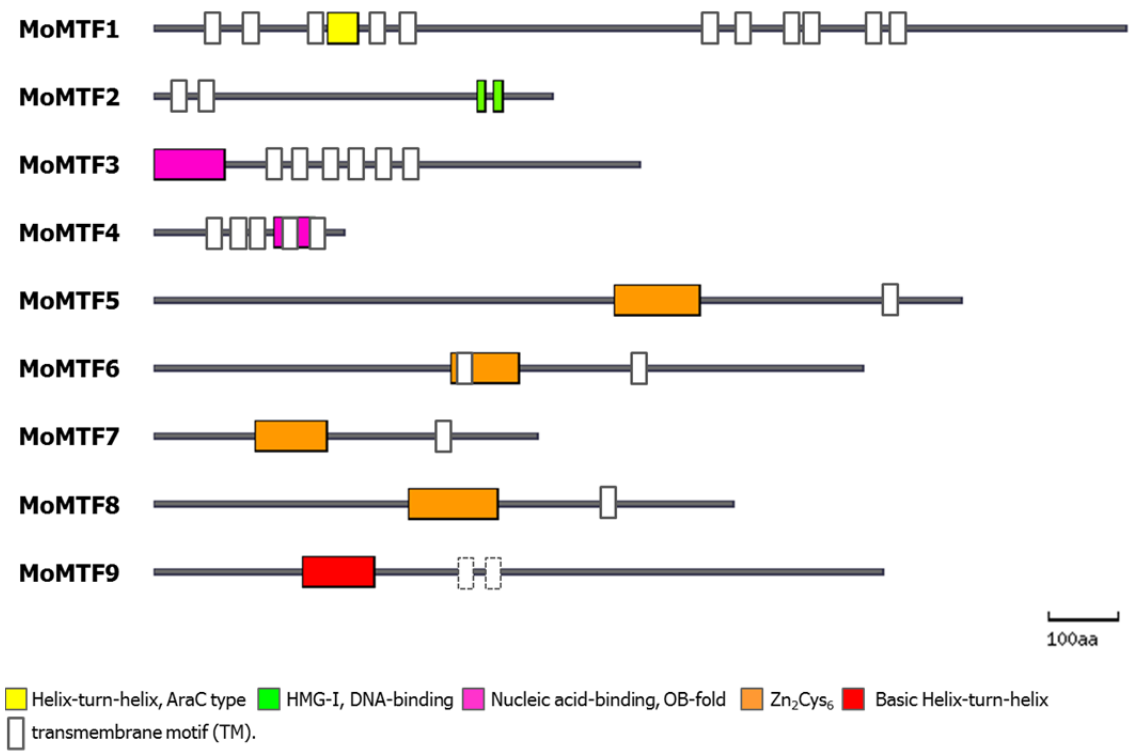

Figure 1. Gene structures of 9 putative membrane-bound transcription factors in *M. oryzae*

II. Phylogenetic analysis of putative membrane-bound transcription factors in fungal kingdom

MoMTFs were analyzed by BLASTMatrix in CFGP (Park et al., 2008b; Choi et al., 2013), a convenient tool for drawing overall picture of homolog relationships in comparative genomics approach. Result showed that MoMTF1 was strongly conserved in every kingdom while homologs with strong similarity with MoMTF6 were distributed in phylum Ascomycota. MoMTF2, 3, 5, 7, 8, and 9 displayed strong conservation within only subphylum Pezizomycotina. Interestingly, MoMTF4 have closely related homologs in species of phylum Basidiomycota, although its conservativeness in subphylum Saccharomycotina and Taphrinomycotina was low (Fig. 2). Phylogenentic tree showed relative distance among MoMTFs and their homologs. One homolog protein of MoMTF6, ASG1, was characterized in yeast, *S. cerevisiae*. It is an activator of stress response genes and essential to grow on non-fermentable carbon sources and stress conditions such as calcofluor white and cycloheximide. MoMTF9 was correlated with SREBP/Ser1 protein and its relatives. Other MoMTFs did not have any experimentally characterized homologs (Fig. 3).

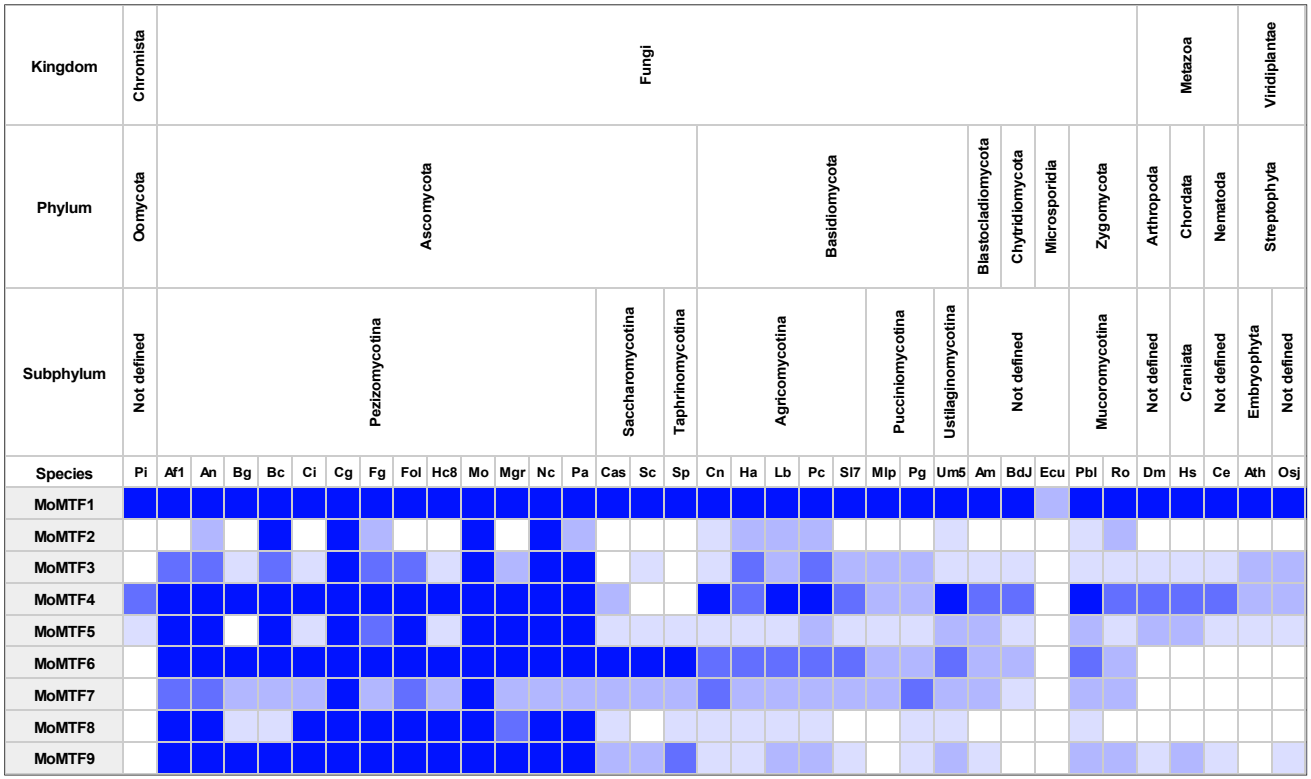

Figure 2. BLASTMatrix analysis of MoMTFs

Dark blue color means strong conservativeness of protein sequence while light blue or white color indicates weak conservativeness or lack of homologs.

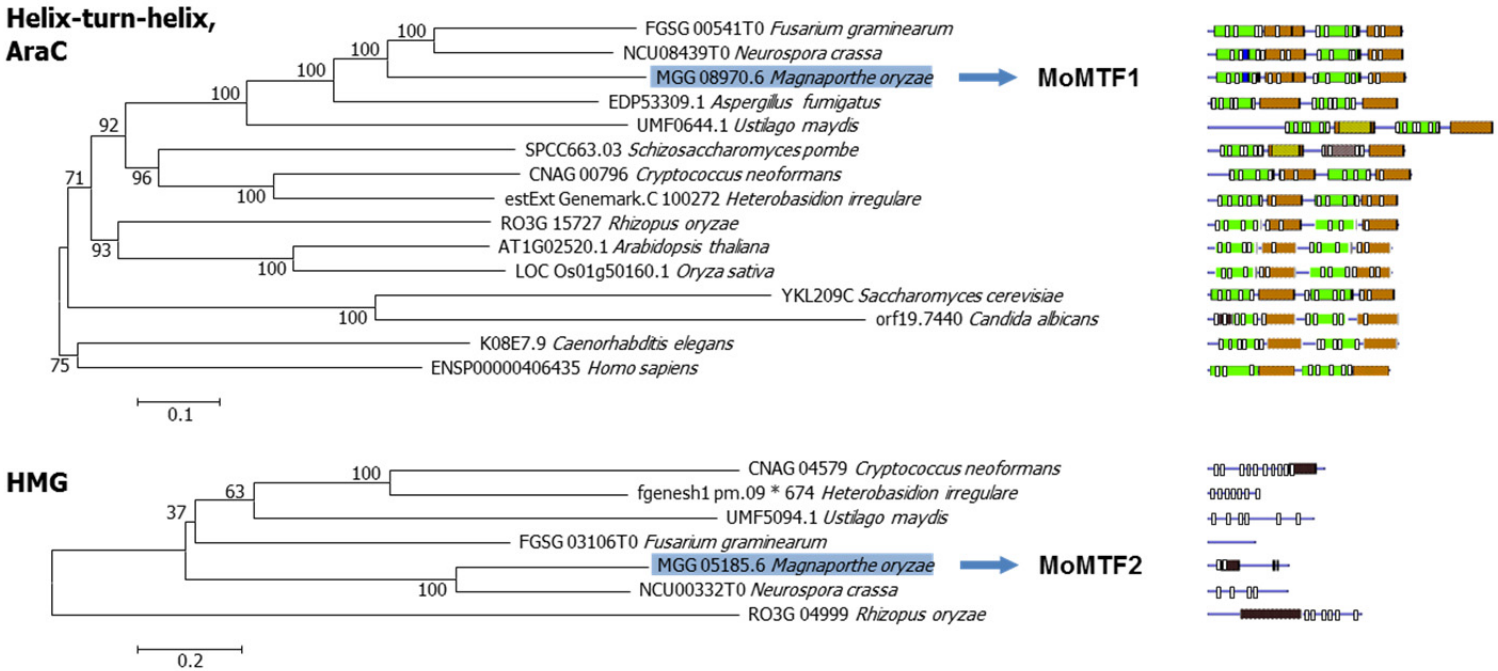

Figure 3. Phylogenetic relationships among MoMTFs and their homologs

Texts at left upper corner of phylogenetic tress indicates TF family. MoMTFs were blue-coloured and homolog proteins already been characterized were red-coloured.

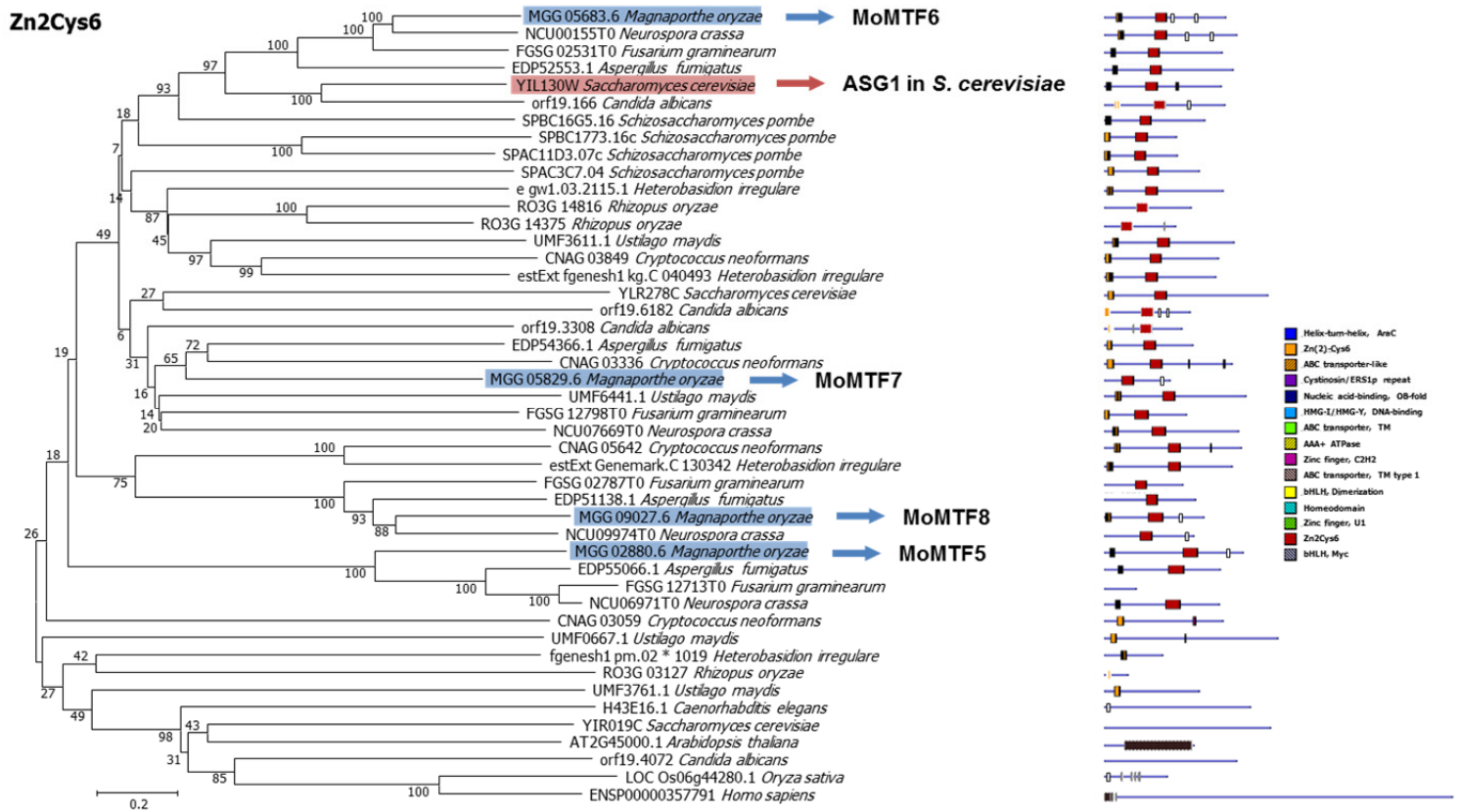

Figure 3. (continued)

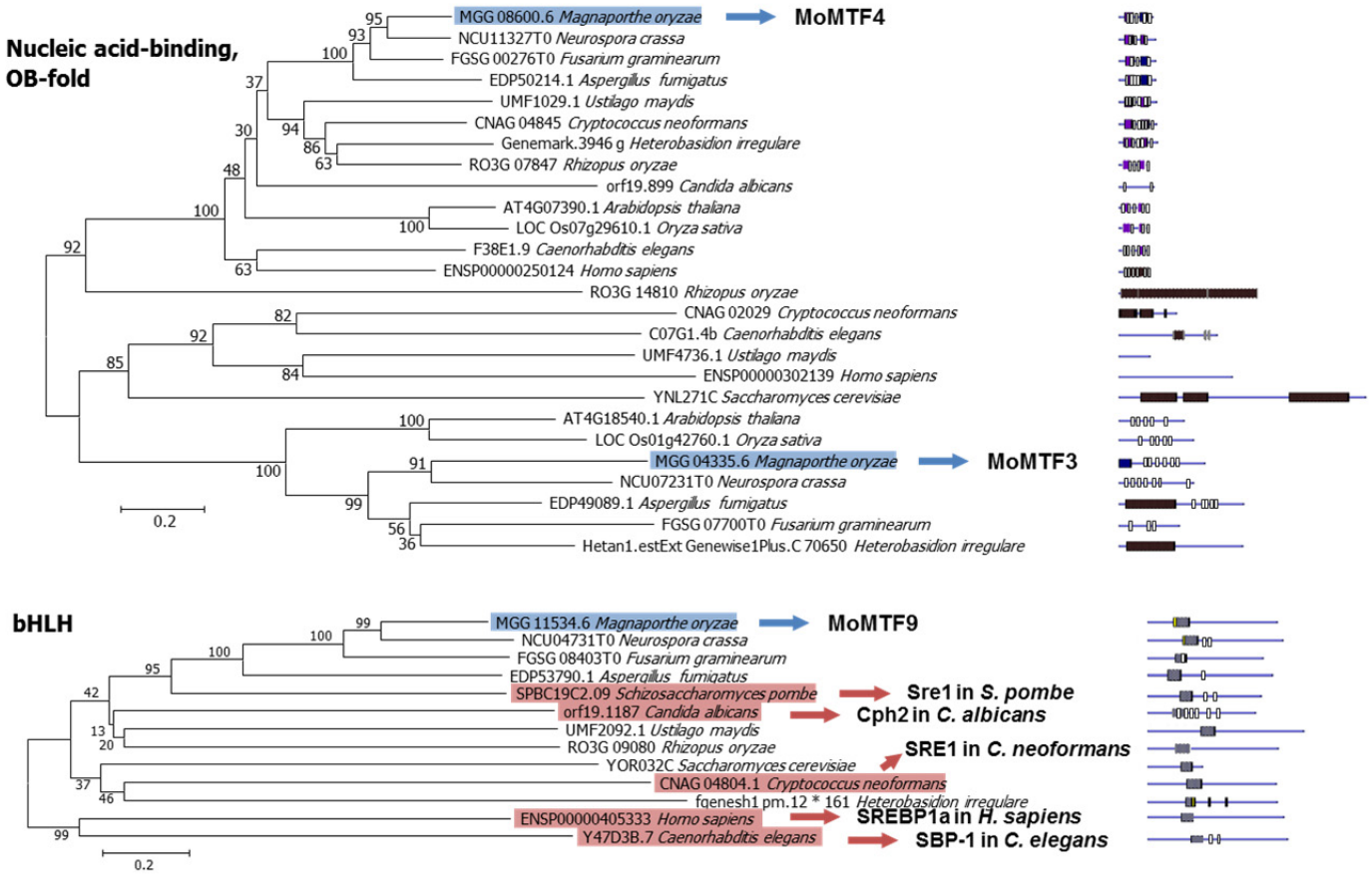

Figure 3. (continued)

III. Disruption of putative membrane-bound transcription factor genes in *M. oryzae*

MoMTF genes in *M. oryzae* were disrupted by targeted gene knockout in which target gene was replaced by *HPH* cassette, a mutant selection marker. I tried to disrupt all *MoMTF* genes and achieved successful mutation in *MoMTF2*, *4*, *5*, *6*, and *8*. Each mutant was confirmed by Southern blot analysis (Fig. 4). Knockout mutants of other genes cannot be produced from the at least three times of fungal transformation and screening of several hundreds of transformants (data not shown).

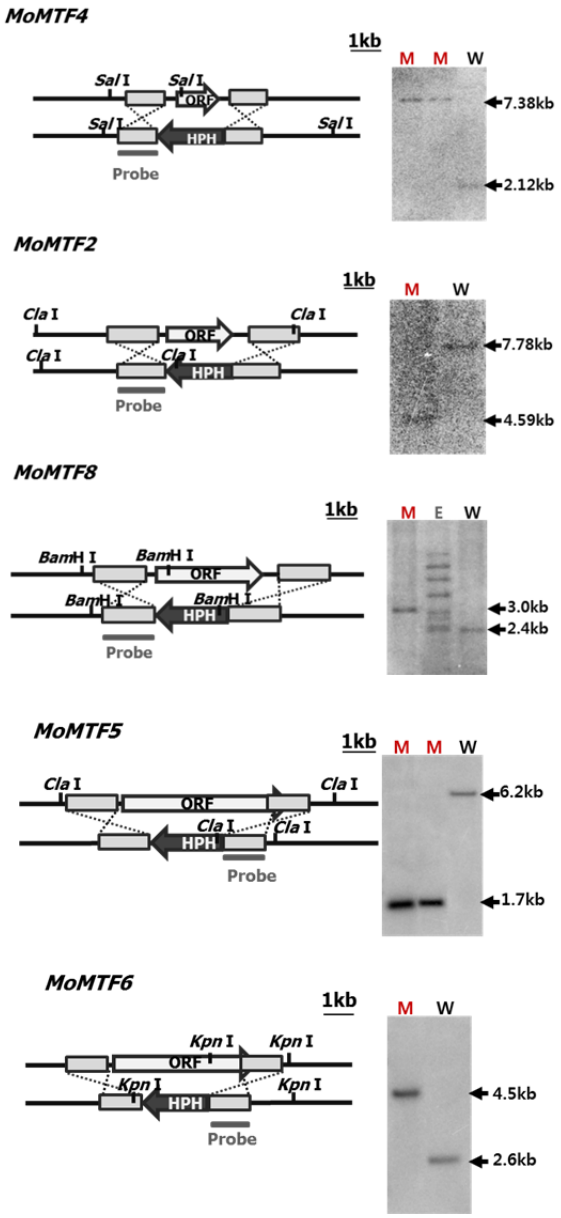

Figure 4. Disruption of MoMTFs

MoMTF genes were replaced with *HPH* cassette by homologous recombination and disrupted mutants were confirmed by Southern blot analysis.

IV. Phenotype analysis of *MoMTF* mutants

Mycological characteristics of *MoMTF* mutants (Δ*Momtf2*, *4*, *5*, *6*, and *8*) were evaluated through observing mycelia growth on solid complete medium, conidia production on solid V8 juice medium, size of conidia, and percentage of conidial germination and appressorium formation. But these phenotypes were indistinguishable between wild type and mutants (Table 4). Mutants could infect the rice seedling and formed lesions similar to that of wild type (Fig. 5). On the contrary, retardation of mycelia growth was detected in Δ*Momtf2*, *4*, *6*, and *8* when they grow on nutritional starvation conditions (Table 5) and abiotic stress conditions (Table 6).

Δ*Momtf6*, a mutant being disrupted homolog gene of *ASG1* in *S. cerevisiae*, however, grew on non-fermentable carbon source and almost identical with wild type on stress conditions such as calcofluor white and cycloheximide.

Table 4. Phenotypic characterization of deletion mutants

Strain	Mycelial Growth (mm)	Conidiation (10^4/ml)	Conidia Morphology (μm)		Conidia Germination (%)	Appressorium Formation (%)
	CM		Length	Width		
Wild Type	62.3 ± 1.9^{A}	28.0 ± 2.9^{ABC}	31.4 ± 1.9^{AB}	9.4 ± 0.6^{AB}	93.3 ± 1.5^{A}	99.7 ± 0.6^{B}
Δ*Momtf2*	59.1 ± 2.3^{A}	$34.0\pm2.4B^{C}$	31.0 ± 1.8^{A}	9.5 ± 0.6^{ABC}	90.7 ± 2.5^{A}	99.3 ± 1.2^{AB}
Δ*Momtf4*	57.4 ± 2.6^{A}	25.3 ± 5.0^{AB}	31.5 ± 2.0^{AB}	9.7 ± 0.7^{C}	89.0 ± 4.0^{A}	100 ± 0.0^{B}
Δ*Momtf5*	61.6 ± 3.8^{A}	18.3 ± 2.1^{A}	31.5 ± 1.9^{AB}	9.6 ± 0.7^{ABC}	90.7 ± 2.1^{A}	100 ± 0.0^{B}
Δ*Momtf6*	56.5 ± 3.4^{A}	40.0 ± 7.8^{C}	31.2 ± 2.0^{A}	9.6 ± 0.6^{BC}	94.3 ± 1.5^{A}	95.9 ± 3.1^{A}
Δ*Momtf8*	60.8 ± 1.5^{A}	32.3 ± 1.7^{ABC}	32.0 ± 2.0^{B}	9.3 ± 0.7^{A}	91.7 ± 3.8^{A}	100 ± 0.0^{B}

Table 5. Mycelial growth under nutritional conditions

Strain	Mycelial Growth (mm)						
	CM	**MM**	**-N**	**-C**	**-C + Lactate**	**-C + Glycerol**	**-C + Sucrose**
Wild Type	67.2±1.8^{A}	69.3±0.3^{C}	66.3±2.3^{B}	72.2±1.4^{B}	46.0±0.0^{AB}	70.3±0.6^{C}	71.2±1.0^{BC}
Δ*Momtf2*	64.7±2.5^{A}	64.0±1.2^{B}	62.2±1.6^{AB}	69.0±0.0^{A}	42.2±2.0^{A}	66.7±1.5^{B}	68.8±1.0^{AB}
Δ*Momtf4*	63.2±2.8^{A}	66.5±2.1^{BC}	61.7±1.5^{A}	69.2±1.4^{A}	43.0±4.4^{AB}	61.3±1.9^{A}	67.7±2.5^{A}
Δ*Momtf5*	65.5±0.5^{A}	66.5±0.4^{BC}	64.8±0.3^{AB}	74.0±1.0^{B}	49.2±1.0^{B}	69.2±0.3^{BC}	72.5±0.5^{C}
Δ*Momtf6*	62.5±0.5^{A}	61.1±1.7^{A}	65.7±2.1^{AB}	68.3±0.6^{A}	41.5±3.3^{A}	68.0±1.0^{BC}	68.0±0.0^{A}
Δ*Momtf8*	65.3±3.1^{A}	64.9±0.9^{B}	62.2±0.8^{AB}	68.7±1.2^{A}	41.7±1.5^{A}	68.8±1.0^{BC}	66.0±1.0^{A}

Table 6. Mycelial growth under abiotic stress conditions

Strain	Mycelial Growth (mm)							
	CM	**Calcofluor white**	**Cyclo-heximide**	**Calcium chloride**	**Manganese (II) chloride**	**SDS**	**Methyl Viologen**	**H_2O_2**
Wild Type	67.2±1.8^{A}	70.3±0.6^{C}	37.5±3.1^{A}	51.8±2.0^{C}	64.2±0.8^{AB}	52.7±2.1^{B}	37.0±2.8^{A}	59.7±1.2^{B}
Δ*Momtf2*	62.0±2.5^{A}	62.8±1.3^{A}	35.7±0.8^{A}	47.2±0.8^{B}	62.5±2.2^{A}	49.3±1.2^{AB}	34.3±0.6^{A}	55.5±0.0^{A}
Δ*Momtf4*	63.7±2.8^{A}	63.7±2.0^{AB}	36.0±0.5^{A}	50.5±0.9^{BC}	63.0±0.0^{AB}	47.8±0.8^{A}	33.0±0.5^{A}	57.0±2.0^{B}
Δ*Momtf5*	65.5±0.5^{A}	67.5±1.8^{BC}	38.2±0.3^{A}	52.0±2.6^{C}	65.2±0.8^{AB}	53.±1.0^{B}	34.5±1.5^{A}	59.0±0.0^{B}
Δ*Momtf6*	62.5±0.5^{A}	63.7±0.6^{AB}	38.7±0.6^{A}	42.5±0.9^{A}	62.5±0.5^{A}	52.±0.8^{B}	38.3±3.5^{A}	54.7±1.5^{A}
Δ*Momtf8*	65.3±3.1^{A}	65.0±2.6^{AB}	36.7±1.2^{A}	49.7±0.8^{BC}	66.3±2.1^{B}	50.0±1.8^{AB}	36.7±2.1^{A}	54.5±0.5^{A}

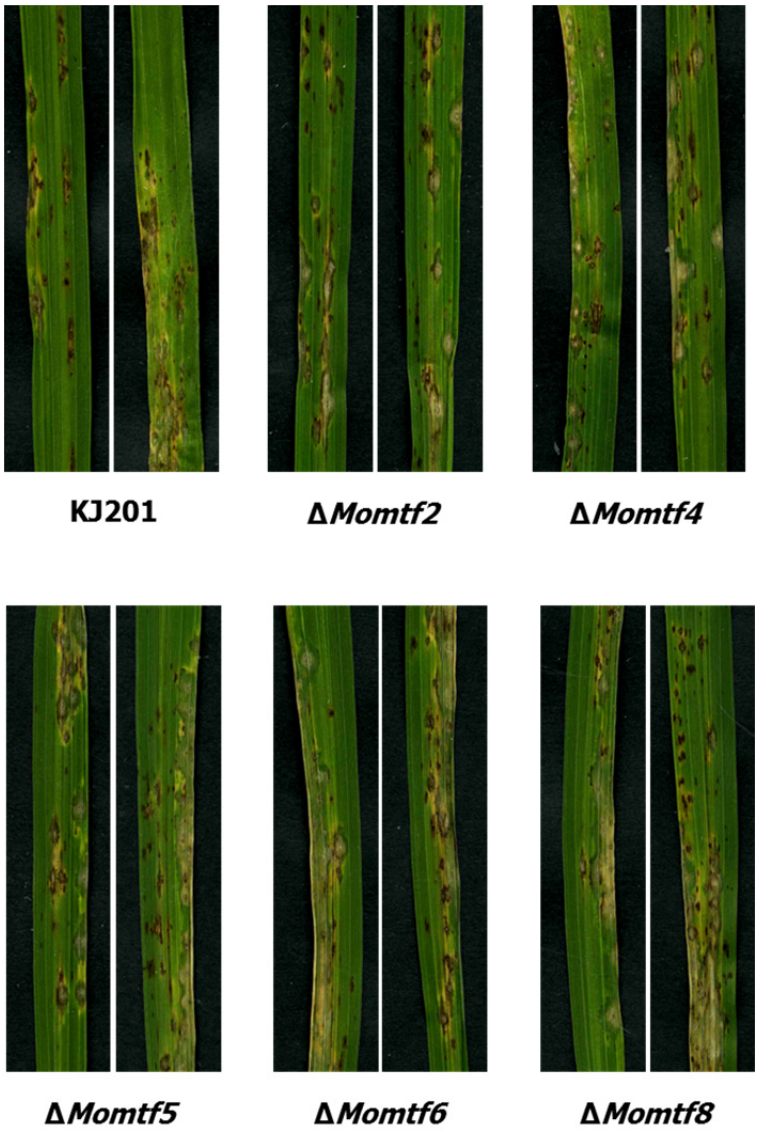

Figure 5. Blast symptoms formed on rice leaves by *MoMTF* mutants
Asexual spores of wild-type and mutant strain were suspended in 250 ppm Tween 20 and sprayed on rice seedling to initiate disease. Disease severity was evaluated on day 7 after inoculation.

V. Gene expression profiling of *MoMTF* genes

Relative gene expressions of *MoMTF2*, *4*, *5*, *6*, *8*, *9* genes were investigated by quantitative RT-PCR. *MoMTF2*, *5*, *6*, *7*, and *9* showed differences in transcription compared to normal vegetative growth condition (CM) when mycelia were treated by several stress and starvation conditions (Fig. 6). *MoMTF6* was highly expressed on osmotic-, ionic-, and heat shock conditions and *MoMTF5* was strongly expressed by exposure to 1 M sorbitol and 0.5 M Lithium chloride. *MoMTF9* was specifically up-regulated on nitrogen-starvation condition.

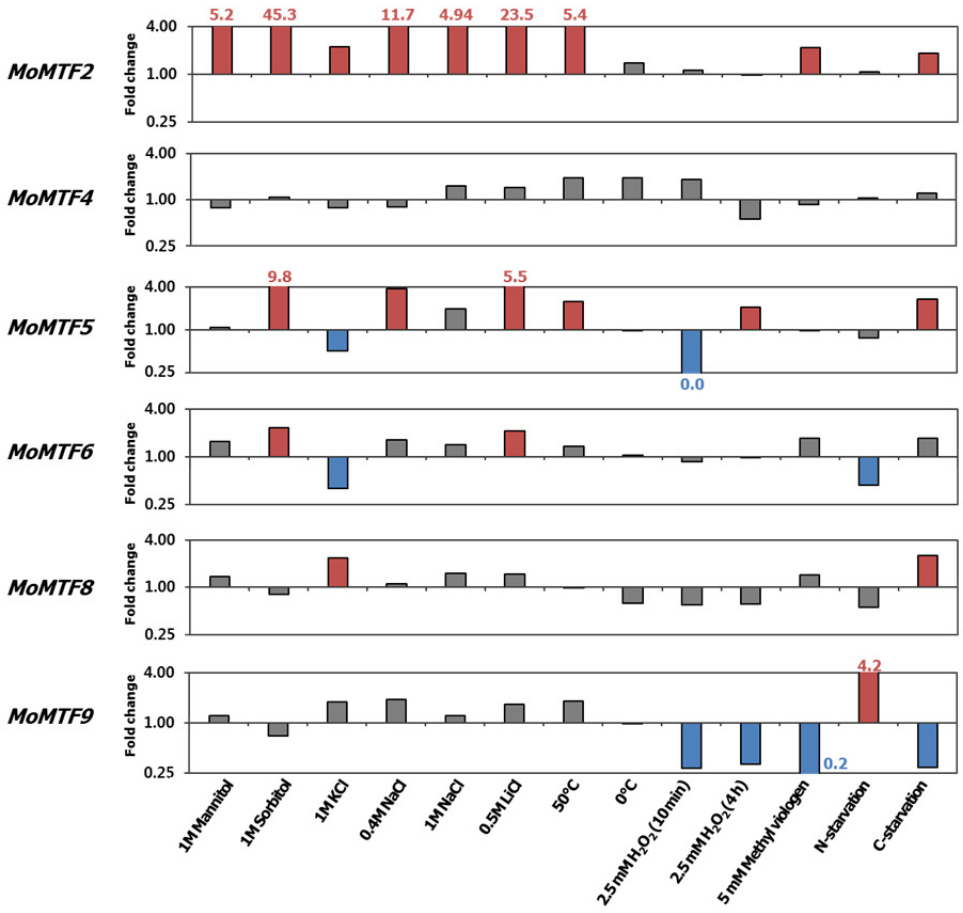

Figure 6. Gene expression analysis of *MoMTF* genes

Relative gene expression of *MoMTF* genes was assessed by quantitative RT-PCR. Red color indicates significant increase of gene expression (>2 of fold change) and blue color means significant decrease of gene expression (<0.5 of fold change). Threshold cycle (Ct) was normalized by $-\Delta Ct = (Ct_{\text{target gene}} - Ct_{\beta\text{-tubulin}})$ and fold change was calculated by $-\Delta\Delta Ct = (Ct_{\text{target gene}} - Ct_{\beta\text{-tubulin}})_{\text{test condition}} - (Ct_{\text{target gene}} - Ct_{\beta\text{-tubulin}})_{CM}$.

VI. EGFP-tagged MoMTF5 protein was localized in plasma-membrane and nuclei

MoMTF5 was selected for EGFP tagging experiment because it contained only one transmembrane motif that is most apart from DNA binding domain and targeted gene knockout of *MoMTF5* was successful. EGFP-MoMTF5 fusion protein was expressed under control of TrpC promoter, a strong and constitutive promoter, because the use of native promoter result in very faint and undetectable GFP signals (data not shown).

EGFP-MoMTF5 was detected at nuclei in *M. oryzae* conidia (Fig. 7). This nuclear localization was confirmed by nuclear staining using Hoechst 33342 (Fig. 8). Interestingly, another GFP signals were detected around the cell of conidia that is thought to be a location of plasma-membrane (Fig. 7). MoMTF5 distributed as a granule form at plasma-membrane and this kind of localization had been reported in previous study about membrane-bound transcription factor in *Arabidopsis thaliana* (Kim et al., 2010).

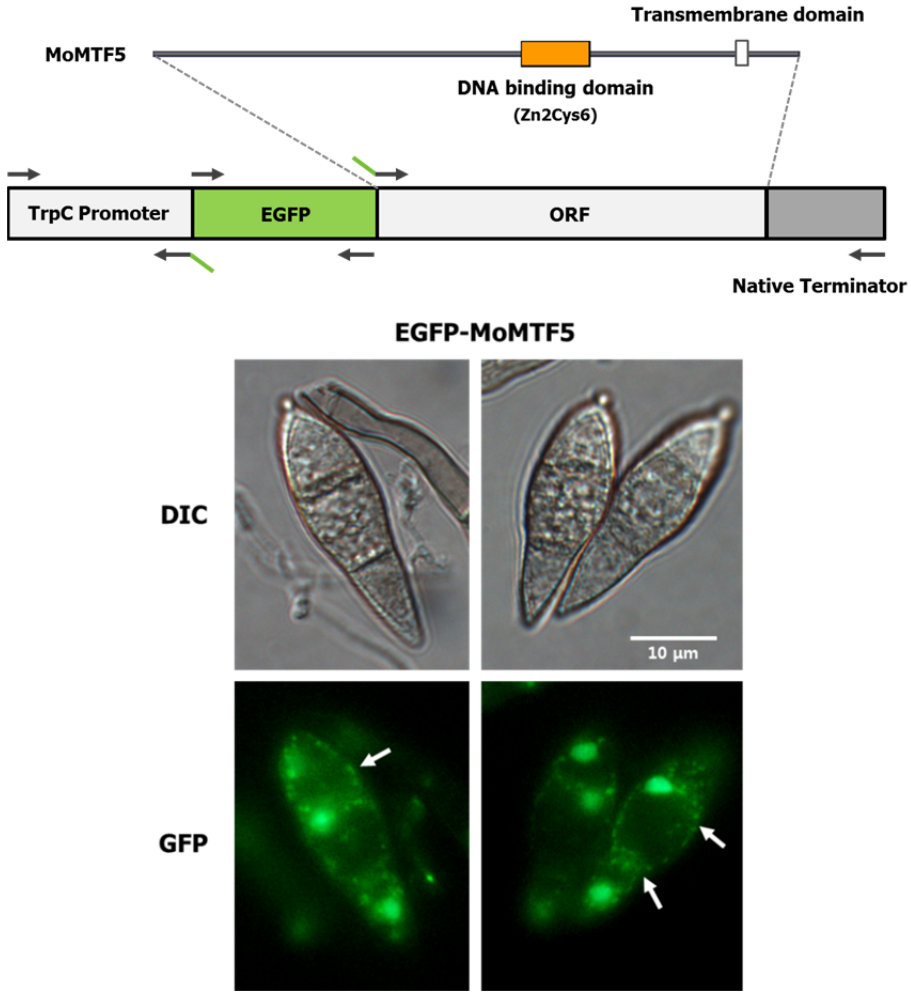

Figure 7. Localization of MoMTF5 tagged with EGFP

EGFP ORF and TrpC promoter were joined at upstream of MoMTF5 gene by double-joint PCR. TrpC-EGFP-MoMTF5 cassette was introduced to *M. oryzae* wild type strain KJ201. Observation of fluorescence in conidia suggested that EGFP-MoMTF5 was located in plasma-membrane (white arrow) and nuclei.

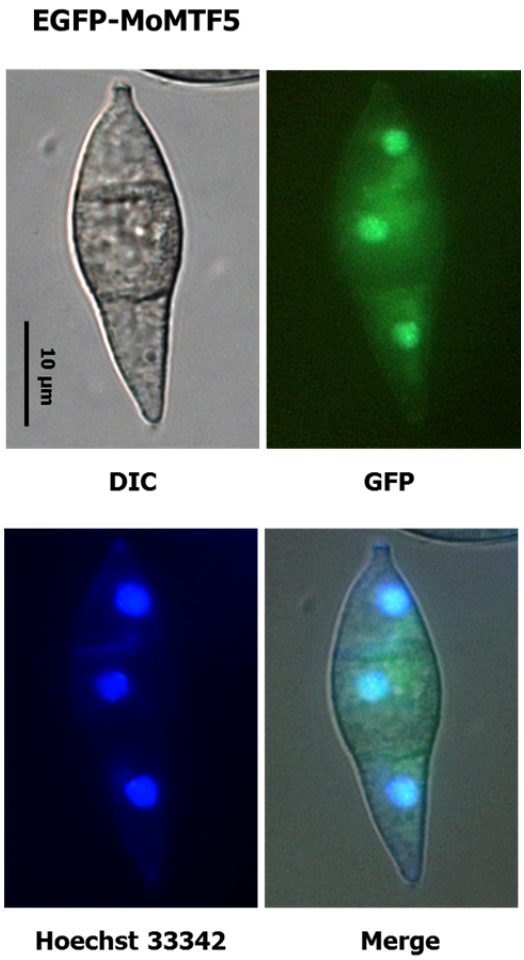

Figure 8. Nuclear localization of MoMTF5

M. oryzae transformant expressing EGFP-MoMTF5 was stained by Hoechst 33342 to visualize nuclei. Merged signals indicated that MoMTF5 was located in nuclei. GFP signals at plasma-membrane were out of focus in this picture.

DISCUSSION

Membrane-bound transcription factors have critical roles in response to intracellular and extracellular changes for homeostasis and adaptation, respectively. MTFs are specific kind of TFs and have transmembrane motifs to bind cellular membranes. This binding restricts MTF to prevent transport to nuclear in non-inducible condition. When specific stimulation occurred, dormant form of MTF is cleaved from membrane and move directly to nucleus. MTFs also have DNA bonding motifs to function in transcriptional regulation by which many TF families are classified. Molecular characterization of MTFs has been well established in plant and animal. However, researches about fungal MTFs reported only limited information. This study, therefore, provides informative data for further analysis of fungal MTFs.

Membrane-bound transcription factors are known as key regulators which response to internal and external changes for homeostasis and adaptation, respectively. Fungal MTFs were not extensively studied and therefore, knowledge is known limitedly in aspect of stress response.

In this work, 9 putative MTFs were identified in *M. oryzae* genome. It counts for 1.9% of total putative TFs in *M. oryzae*, that is quite smaller than *A.*

thaliana (4.5%) and similar to *O. sativa* (2%) (Kim et al., 2010). More than half of putative MTFs in *M. oryzae* have 2 or more transmembrane motifs predicted by TMHMM 2.0 server otherwise almost of MTFs in *A. thaliana* and *O. sativa* contained only one motif (Kim et al., 2010). Four out of 9 putative MTFs in *M. oryzae* were Zn2Cys6 family TFs, the fungal specific TFs, so they probably function in fungal specific mechanisms.

Phylogenetic analysis showed possible ortholog relationships about MoMTFs. But some orthologs of MoMTFs does not have transmembrane motif. It may due to prediction errors in TMHMM 2.0 server or gene modification during evolution. ASG1 in *S. cerevisiae* is a probable homolog protein to MoMTF6 according to phylogenetic tree. However, mutant characterization indicated that their roles are quite different although both were related to stress response. MoMTF9 is highly probable homolog to a SREBP protein in animal and Sre1 protein in fungi which are well-known regulator for sterol homeostasis/synthesis. This will be a valuable discovery for further research.

As a result, five putative MTF genes (*MoMTF2*, *4*, *5*, *6*, and *8*) were disrupted and characterized. Knockout mutants were indistinguishable to wild type in mycelial growth on complete medium, conidiation, conidial morphology, conidial germination, appressorium formation, and

pathogenicity. It implied that these MoMTFs were not required in routine growth condition and fungal development. On the other hand, Δ*Momtf2*, *4*, *6*, and *8* showed growth retardation in various starvation and stress conditions. Gene expression profiling also showed that some *MoMTF* genes were dramatically up regulated on osmotic-, ionic-, oxidative- and starvation stress conditions. It suggested the function of MoMTFs in stress- and environmental change adaptation.

Cellular localization of MoMTF5 was confirmed by EGFP tagging. As expected, it was located at plasma-membrane forming granule like distribution and nuclei.

To conclude, although *MoMTF2*, *4*, *5*, *6*, and *8* were not essential for normal growth, development, and virulence, they related to environmental fitness and may play a pivotal role for survival in transilient circumstance including various stresses and starvation.

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벼 도열병균 막결합전사인자 유전자들의 기능 규명

정 수 빈

초 록

막결합전사인자, membrane-bound transcription factor(MTF)는 다른 유전자의 발현을 조절하는 전사인자 가운데 세포 내 외 인지질 막에 결합되어있는 것들을 말한다. 이들은 막에 결합하여 휴면상태로 존재하다가 환경변화나 스트레스와 같은 특정한 조건에서 발현되게 되는데, 이러한 발현 기작은 다른 전사인자들의 조절 기작과는 다른 특징으로써, 이를 통해 다른 전사인자들과는 구분되는 역할을 할 것이라 기대된다. 하지만 이렇게 중요한 단백질임에도 불구하고 벼 도열병균(*Magnaporthe oryzae*)을 포함한 식물 병원

성 곰팡이에서는 그 기능이 연구된 바가 없었다. 특히 벼 도열병균은, 벼의 전 부분, 전 생육기에 감염하여 전 세계적으로 심각한 식량 손실을 일으키는 경제적 중요도가 높은 곰팡이이므로 이에 관련한 막결합전사인자 연구는 필수라 생각되었다. 본 연구에서는 곰팡이 전사인자 데이터베이스를 사용하여 벼 도열병균의 유전체로부터 총 9개의 막결합전사인자들을 밝히고, eGFP tagging기법을 이용하여 실제로 막에 존재하는지에 대한 여부를 확인하였다. 또한 9개의 MTF 중 5개의 유전자 삭제변이체를 만들었고 이를 이용하여 유전자 기능을 알아본 결과, *⊿Momtf2, 4, 6, 8* 이 다양한 스트레스 조건에서 야생형 대비 균사생장에 지연을 보임으로써, 벼 도열병균의 막결합전사인자가 스트레스 조건을 포함한 여러 가지 환경변화 적응에 관여한다는 사실을 알 수 있었다. 위 결과들로 막결합전사인자의 기능을 유추함으로써 석사학위 논문으로 가치가 충분히 있다고 여겨진다.

주요어: 벼 도열병균, 막결합전사인자

학　번: 2011-23536

감사의 글

2011년, 설레임으로 시작했던 석사과정이 2년이라는 시간이 흘러 졸업을 앞두고 있습니다. 그동안 부족한 저에게 많은 도움과 힘을 주셨던 분들께 감사의 인사를 드리고 싶습니다.

먼저, 언제나 열정적인 에너지로 저희를 이끌어주시고 제가 끝까지 배움에 대한 호기심을 가질 수 있게 지도해 주신 이용환 선생님께 진심으로 감사의 인사를 드리고 싶습니다. 주신 가르침을 언제나 마음에 새겨 훌륭한 제자가 되도록 하겠습니다. 그리고 식물미생물학에 대해 양질의 지식으로 배움의 길을 인도해주신 이인원 선생님, 박은우 선생님, 김영호 선생님, 황인규 선생님, 가종억 선생님, 김국형 선생님께 감사 드립니다. 미숙하고 부족한 저를 오랜 기간 동안 지켜봐주시고 석사과정을 시작할 수 있게끔 많은 용기와 도움을 주셨던 임용표 교수님께도 감사의 인사를 드리고 싶습니다. 또한 대학원 기간 동안 여러 가지 도움을 주었던 균병학 연구실 멤버들에게도 감사 드립니다. 2년 동안이지만, 많은 것을 배울 수 있었고 보다 좋은 방향으로 성장할 수 있었습니다.

그리고 항상 저의 뒤에서 저를 응원하고 지지해주는 우리 가족에

게 마음 속 깊은 고마움을 전하고 싶습니다. 세상에서 가장 존경하는 우리 아빠와 엄마, 아직도 걱정만 끼쳐드리는 것 같아 딸은 아직도 갈길이 멉니다. 효도할게요. 사랑합니다. 부족한 언니를 가장 잘 이해하고 따라주는 내 반쪽이자 분신인 귀여운 동생 혜원아, 고맙고 사랑해!

2년 동안, 내게 버틸 수 있는 힘과 엔도르핀을 주었던 깜찍한 노디. 언니가 오히려 너에게 배웠단다. 정말 고마워~네 덕분에 힘들었던 시간들도 이젠 추억이 되었어. 애정하는 내 친구 혜성아~ 우울하다가도 널 만나면 까르르 웃을 수 있었고 네 배려와 따뜻한 마음씨에 난 아직도 감동 중이야~우리 할머니 되어서도 함께 하자~언제나 고마워~^^* 두서없는 이야기도 본인 일처럼 들어주던 짱형, 고마워요~그리고 미안하고 고마운, 평생 잊지 못할 은인이신 겸충씨. 이젠 제가 은혜 갚을 차례지요.

동병상련의 마음으로 나를 잘 이해해준 선아, 완전 매력 있는 우경이, 그리고 이젠 오랜친구가 된 명숙아, 나 아줌마 되어도 놀아줄거지?

마지막으로 넓고 깊은 마음으로 조용하게 나의 마음을 움직이는 평생 동반자가 될 분에게 세상에서 가장 큰 믿음과 사랑을 드립니

다. 한결 같은 모습으로 곁을 지켜주어서 큰 힘이 되었어요.

미처 언급하지 못한 분들을 포함한 많은 분들의 도움으로 석사 과정을 마무리 지을 수 있었습니다. 앞으로 더욱 발전하는 사람으로 성장하겠습니다. 다시 한 번 진심으로 감사드립니다.

정수빈 올림