Functional identification of a transcriptional factor, Myb2 in *Giardia lamblia* by comparative proteomic analysis

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Directed by Professor Soon-Jung Park

The Master's Thesis submitted to the Department of Medical Science, the Graduate School of Yonsei University in partial fulfillment of the requirements for the degree of Master of Medical Science

So Hyun Bang

June 2007

This certifies that the Master's Thesis of
So Hyun Bang is approved.

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The Graduate School
Yonsei University

June 2007

ACKNOWLEDGEMENTS

It was not easy for me to study in the master program of Cellular biology and pharmacology in the last two years; however, it gave me big challenges to learn many things and to grow up my mind through the time for my life.

I' d like to express my heartfelt thanks to Professor Soon-Jung Park who always gave me the right thoughts and directions with the interest and encouragement and to Professor Tai-Soon Yong and Professor Seungwoo Park who gave me great help and made me a scientist of widen my knowledgeable view. Also I appreciate many advices from Professor Myeong Heon Shin.

From the last 2005, I met good people such as Miss Juri Kim, Mrs. Woo Hyang Kim, Mrs. So-Yong Na, Miss Do-Yeon Kim, Sung Young Goo, Sung Su Bae, and One Hwang who gave me advices and supports while I started work in the laboratory. Young Ah Lee, Myung Hee Lee, Sun Jin Eun, Kyung Ah Kim, and Deul le Min shared whenever I experienced both joy and sorrow. Kwang Hyun Shin, the classmates, always gave me big help during the master program. I thank to the entire classroom member from the bottom of my heart.

I want to show my gratefulness to Mi Na Ju and Mi-Kyung Song, always having a

strong believes in me and standing by me. In addition, I thank to Sanghee Shin, Han-Na Lee, Yu-Jin Kim, Min Jeong Kim, Hey-Jin Choi, Ho Sung Park, Bong Gyu Jee, Jung Hun Nam, Se-Hoon Gu, and Min Kyung Goo because whenever I meet them, I am happy all the time and I got good advices from them. Also I deeply return big thanks and express my love to Jae Young Cho who think and love me the top priority all the time.

I can not find the words to thank you: my Father who gives me the unstinted love than no one ever does, my the eldest sister and brother-in-law, who took a good care of while studying, the second eldest sister who gave me a big support, the third sister, called “kkoma sister”, and the brother-in-law who gave me a console words and encouragement when I faced the hard time. Nephews as Tae-Hyung, Jun-Hyung, Nam-Ju, and niece Min-Ju made me laugh and happy. Lastly, I devote this paper to my mother who looking after and loving me forever in Heaven.

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Functional identification of a transcriptional factor, Myb2 in *Giardia lamblia* by comparative proteomic analysis

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Giardia lamblia, a human pathogen causing diarrheal ourbreaks, has a life cycle composed of trophozoite and cyst. Previous study on encystation, transformation of trophozoite into cyst, identified several encystation-induced genes including *myb2*, which encodes a putative transcriptional factor. Using a transgenic *G. lamblia* overexpressing Myb2 protein, we screened target gene(s) controlled by Myb2 protein.

A Myb2-overexpression plasmid, pmyb2.pac was constructed by replacing the *gfp* gene of pGFP.pac with the *myb2* gene. Using a two dimentional gel electrophoresis, proteome of *G. lamblia* trophozoites carrying pmyb2.pac was compared with that of *G. lamblia*, which did not carry any plasmid. In addition, the proteome of encysting cells was compared with that of trophozoites. Ten of the increased protein spots in extracts of *G. lamblia* with pmyb2.pac and 18 increased spots in the encysting cells were identified as up-regulated proteins, which could be categorized as cytoskeletons, metabolic enzymes, cell-cycle specific kinases, stress resistance proteins, a protein involved in translation and hypothetical open reading frames. Among them, eight genes were cloned and examined for their expression pattern during encystation as well as in transgenic *G. lamblia* overexpressing Myb2 by real-time PCR. Expression of the *cwp1* gene (encoding cyst wall protein 1) was monitored as a positive control for encystation, whereas expression of the *tim* gene (triose-1-phosphate isomerase) was also measured by real-time PCR as a loading control for the RNA amount. Expression of the *cwp1* gene was increased 33-fold upon induction to encystation, whereas the *tim* gene was expressed in a constitutive mode during encystation. Two stress response genes encoding heat shock protein 70 and heat shock protein 90 and 2 hyphothetical genes showed increased expression (2- to 7-fold) during encystation. In

Myb2-overexpressing *G. lamblia*, only the genes for heat shock protein 70 and a hypothetical protein (ORF17060) demonstrated 3- to 4-fold increase in the level of their transcripts. Gel-shift assays indicated that the recombinant Myb2 protein bound to the promoter regions of the *cwp1* gene, ORF17060, and G2-specific kinase fin1. These results suggest the genes for heat shock protein 70 and ORF17060 as targets controlled by Myb2 protein.

Key words : *Giardia lamblia*, Myb2, encystation

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Ⅰ. INTRODUCTION

Giardia lamblia, a human pathogen causing diarrheal disease [1], is important protozoa. Based on phylogenic analyses of its ribosomal RNA sequence and various protein coding sequences [2-6], *G. lamblia* is an interesting microorganism regarding its phylogenetical position as a member of the earliest lineage of the *Eucarya* during evolution [7-10]. It has a life cycle composed of two forms [1], like *Entamoeba*, the

proliferative trophozoite and the non-proliferative, infectious cyst [1,11]. Infection occurs upon peroral ingestion of cyst in contaminated water or food [12-13]. Attachment of trophozoites to epithelial cells of small intestine is essential for *Giardia* colonisation in the intestine and, most likely, also results in damage of the intestinal epithelium [14-16]. Following excystation in proximal duodenum of the host [1], cysts release an excyzoites, which only represents a transient stage of the life cycle and immediately divide into four trophozoites [11]. During this initial proliferation step, the trophozoites form adhesive ventral disk [17]. Adherence of the trophozoites to the intestinal wall and, more specifically, to the microvillous brush border of enterocytes, basically occurs through the function of the ventral disk but specific receptor-ligand interaction also seem to be involved [18-19].

Excystation and encystation are essential for the life cycle of *G. lamblia*, and for its survival within and out of the host [1]. Although encystation can be involved *in vitro* by modeling physiological stimuli (elevated pH), its regulation is not understood [20-23]. The distinct structural differences between these two forms imply that a series of involved genes are probably differentially expressed during these two processes. Previous studies on encystation identified several encystation-induced genes, such as *cwp1, cwp2* [24], *bip/grp78* [25], *enc1*, and *enc6* [26]. In addition, Knodler et al. [27] reported

that the expression of *gln6pi-b* for glucose-6-phosphate isomerase composing biosynthetic pathway for the cyst wall, was also induced during encystation. Synthesis and assembly of the extracellular cyst wall are major hallmakers of this important differentiation [21-23, 28]. As well as Sun et al [29] reported two *myb* homologous genes, *myb1* and *myb2* by searching the *G. lamblia* database using the DNA binding domains of Myb proteins of other organisms as a clue. They found that the expression of the *myb2* gene was induced during encystation, whereas the expression of *myb1* was constitutive.

Transcriptional control is a major mechanism whereby a cell or organism regulate its gene expression. Sequence-specific DNA-binding transcription regulators, one class of transcription factors, play an essential role in modulating the rate of transcription of specific target genes [30].

Myb family transcription factors are important in regulating developmental processes in diverse organisms including fungi, plants, and mammals [31-34]. In mammals, c-Myb can function as a transcriptional activator and regulate differentiation and cell cycle progression [35-36, 33]. The absence of Myb from eubacteria indicates that the Myb domain evolved after the eukaryotic divergence [37]. The family of Myb proteins exhibits substantial homology in their Myb domains, but not in other

regions [37]. c-Myb and many other Myb proteins, contain three characteristic imperfect 52 amino acid repeats in their Myb domains [37-38]. v-Myb, a truncated version of c-Myb found in a leukaemia retrovirus, like most plant Mybs, contains only two repeats, R2R3, the part essential for DNA binding [32, 38-40].

The lack of clear giardial homologues of many known basal transcription factors supports the idea that *Giardia* diverged early and that it may represent a transition during the evolution of eukaryotic transcription systems [41]. Moreover, many aspects of giardial gene are unusual. For an example, no consensus TATA boxes or CCAAT boxes or other *cis*-acting elements characteristic of higher eukaryotic promoters has been observed in the promoters of many giardial protein-coding genes. *Giardia* has a highly divergent TATA-box binding protein and lacks most of the canonical RNA Polymerase II transcription factors [41]. The transcription start sites of many constitutive and encystation-induced giardial genes are within AT-rich sequences. The AT-rich sequences are essential for promoter activity and play a predominant role in determining the positions of the transcription start sites [24, 27, 42-46]. They are functionally similar to the initiator element in higher eukaryotes. The giardial promoter regulatory mechanism may be unusual because very short 5'-flaking regions (<65 bp) are sufficient for the expression of most genes [42, 45-46].

We performed a construction of Myb2 overexpressing plasmid in *G. lamblia*, an examine of increased target genes expression in overexpressing Myb2, and investigation of binding promoter region of acquired Myb2 target genes and Myb2 protein to role of increased genes during encystations.

This study will find a regulated target gene of expression by Myb2 through comparative proteomic analysis and provide information about encystation, one of the two composed with life cycle.

Ⅱ. MATERIALS AND METHODS

1. Culture and encystation

Trophozoites of *Giardia lamblia* WB strain were cultured for 72 hr in TYI-S-33 (2% casein digest, 1% yeast extract, 1% glucose, 0.2% NaCl, 0.2% L-cystein, 0.02% ascorbic acid, 0.2% K_2HPO_4, 0.06% KH_2PO_4, 10% calf serum, 0.5 mg/ml bovine bile, pH 7.1) medium at 37℃. Trophozoites were transferred into an encystation medium containing 10 mg/ml bovine bile at pH 7.8. After 6, 10, 12, and 15 hr-incubation in the encystations medium, the cells were harvested by centrifugation at 3,000 rpm for 15 min at 4℃.

2. Plasmid construction

A plasmid, pmyb2.pac were constructed by replacing the *gfp* gene of pGFP.pac [47] with the *myb2* gene. The *myb2* gene were amplified by PCR using two primers (F : 5'-TTAGCGGCCGCAGGGTAGCTTCTACG-3' and R : 5'-CCAGCCATGGATGTT ACCGGTACCTTCTCAG-3') from genomic DNA of *G. lamblia* WB and then were cloned into *Nco Ⅰ*, *Not Ⅰ* site of pGFP.pac.

3. Transfection

Trophozoites of *G. lamblia* were grown for 72 hr in TYI-S-33 medium and incubated on ice for 15 min. The cells were harvested by centrifugation by at 3,000 rpm for 20 min at 4℃, resuspended in serum-free TYI-S-33 medium and then transferred to electroporation cuvettes. Twenty five μg of DNA were added into the prepared cells and incubated on ice 5 min. After electroporation was carried out under the condition of 350 volt, 1000 μF, 720 Ω, the cells were cultivated in TYI-S-33 medium for 24 hr at 37℃. Transfectants were selected by adding puromycin at the final concentration of 100 μM.

4. Western blot analysis

The recombinant Myb2 (rMyb2) protein was expressed as a histidine-tagged form in *Escherichia coli* BL21 (DE3), purified by Ni^{+}-NTA chromatography, and then used to immunize rats to make specific antibodies. Crude lysates were prepared from *G. lamblia* without any plasmid or *G. lamblia* with pmyb2.pac in a lysis buffer (50 mM Tris-HCl, pH 7.4, 25 mM EDTA, pH 8.0, 650 mM NaCl, 5% Triton X-100) a containing protease inhibitors (1X PMSF, 0.7 μg/ml pepstatin A, 0.5 μg/ml leupeptin, 0.5 mg/ml TLCK). Prepared lysates were then separated by SDS-PAGE, and then

transferred to nitrocellulose membrane. The membrane was serially incubated with anti-Myb2 antibodies (1:500D) and goat alkaline phosphatase-conjugated anti-rat lgG (1:1,000D). An immunoreactive protein was visualized by incubating the membrane with NBT/BCIP.

5. Two-dimentional polyacrylamide gel eletrophoresis

Harvested *G. lamblia* cells were resuspended in a buffer (20 mM Tris-HCl, pH 6.8), and homogenized on ice, and centrifuged at 16,000 rpm for 10 min at 4℃. The soluble proteins were precipitated with 30 min incubation on ice with 0.1% TCA (trichloroacetic acid). The precipitated protein was resuspended in a lysis buffer (8 M urea, 4% CHAPS). Three hundred micrograms of the proteins was loaded onto the pH 4 to 7 immobilized pH gradient (IPG) strips by in-gel rehydration using IPGPhor (24 cm; Amersham Pharmacia Biotech, Piscataway, NJ, USA). Isoelectric focusing was carried out successively at 300 V for 3 hr, 3,500 V ~ 100,000 V for 1 hr. After isoelectric focusing, strips were incubated in an equilibration buffer 1 (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, BPB, and 2% DTT) for 15 min, and then in an equilibration buffer 2 (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, BPB, 2% DTT, and 2.5% iodoacetamide) for 15 min. The equilibrated strip

was placed on a polyacrylamide gradient slab gel. Separation was continued at 10 mA/gel in a running buffer (25 mM Tris, pH 8.8, 198 mM glycine, and 0.1% SDS) until BPB reached the bottom of the gel. After electrophoresis, the protein spots were visualized with a modified silver staining protocol that is compatible with MALDI-TOF MS. Silver stained gel was scanned using densitometer 800 (Bio-Rad, Heracules, CA, USA). The digitalized image was analyzed using PDQUEST software (V. 6.1; Bio-Rad, Heracules, CA, USA).

6. Real-time PCR

Eight genes were chosen, whose expression were up-regulated either in *G. lamblia* carrying pmyb2.pac or in the encysting cells. Using Trizol, RNA was prepared from trophozoites, or the encysting cells at 6, 10, 12, or 15 hr after being induced to encystation. In addition, RNA was isolated form *G. lamblia* carrying pmyb2.pac. Five micrograms of RNA was converted into cDNA using StrataScript cDNA synthesis kit (Stratagene, La Jolla, CA, USA). Eight pairs of forward and reverse primers used for real-time PCR, were designed using Primer Express 2.0, and listed in Table 1. Real-time PCR was performed using SYBR Green PCR master kit (ABI, Warrington, UK) with a pair of the primer sets. Conditions for real-time PCR were as

follows : pre-incubation at 50℃ for 2 min, 95℃ for 10 min, and 25 cycles of amplification (94℃ for 30 sec, 55℃ for 1 min, and 72℃ for 2 min).

Table 1. Primer sequences for real-time PCR

Name of putative protein	Sequences (5'→3')
Myb2	F CACCAGAAGAAGACGAGGCTTT
	R TGGCGACGTTCTTCCAGTTT
TIM	F CGAAAGTGGTTTGCGGAGAA
	R TTCTCGCAGTTGCTTCCATTG
CWP1	F GGCTTTCGGGAGACTCTATTTG
	R ATGTTCTCCGGGATGGTACCT
Never in mitosis gene a related kinase 1	F TCTGTGCAAGCGGAAAGATTG
	R GTCTCTGGCAGCGATCATGAG
Chaperonin	F GCAGTTCATGCCAGAGCCATA
	R AACTCCTGCCTGGACGTTGT
myo-inositol 1-phosphate synthase	F AGGCACCACTTCCTCGAACA
	R CTGGAGTCCTGCTATTGCTCTCA
heat shock protein 70	F ACGTCTGGCACCAACATGAC
	R AGATCCTGATTGTAACGGTTGTCT
heat shock protein 90	F GACATGTGGGACGCTCAGAA
	R GTAAAGGAGTCTCGCACACATACA

G2-specific kinase fin1	F	ATCGGTGCAACAGCCTTGAT
	R	CGTTCTGGGCTGCAAACAT
ORF 8329	F	GAAGGCGATCTTCAAGAACACA
	R	TTCGGGTGCAGAAAGAAGGT
ORF 17060	F	TGTGAGCTCTCGCCCAAAG
	R	TAGCAGGGCGGTAACAACCT

7. Gel-shift assay

Genes were chosen, whose expression were increased in trophozoites of *G. lamblia* carrying or during encystation in real-time PCR. DNAs tested for rMyb2 binding were cloned using primer in Table 2. Using timF and timR primers, the promoter region of the *tim* gene was cloned, and used as a control to examine of rMyb2 binding. The promoter DNAs were labeled with γ-^{32}P ATP using T4 DNA kinase. Binding reaction was carried out in a reaction buffer (3 mM HEPES, pH 7.9, 0.6 mM $MgCl_2$, 2 mM KCl, 0.2 mM EDTA, 0.1 mM DTT, 2% glycerol, 2 μg poly dI-dC). The binding mixture was incubated for 30 min at 37℃ and then separated on a 5% native polyacrylamid gel.

Table 2. Primer sequences for Gel-shift assay

Name of genes	Sequences (5'→3')
tim	F CGAAAGTGGTTTGCGGAGAA
	R TTCTCGCAGTTGCTTCCATTG
cwp1	F GGACCTCGGGTCTCCAGAGG
	R AGGAGAGCGAGCATCATCCC
ORF17060	F AGGTGGTAGACTCGCATACCC
	R CGACTGCGTTGAAACGACCAT
G2-specific protein kinase fin1	F CAGGAAGCAGAGATCAAGCG
	R GGTGCTCCATCGGCTTGTGA

Ⅲ. RESULTS

1. Construction of a Myb2 overexpression plasmid of *G. lamblia*

We constructed a plasmid, pmyb2.pac, in which the *gfp* gene of pGFP.pac [47] was replaced by the *myb2* gene (Fig. 1). The resultant plasmid was transfected into trophozoites of *G. lamblia* WB. The transfectants selected by puromycine resistance were examined for the expression of Myb2 protein by western blot analysis using polyclonal antibodies specific to Myb2 (Fig. 2). Cell extracts prepared from *G. lamblia* carrying pmyb2.pac demonstrated an immunoreactive band of 62 kDa, whereas it was missing in the extracts prepared from *G. lamblia* without plasmid. This result clearly indicates that Myb2 protein overexpresses in *G. lamblia* trophozoites carrying pmyb2.pac.

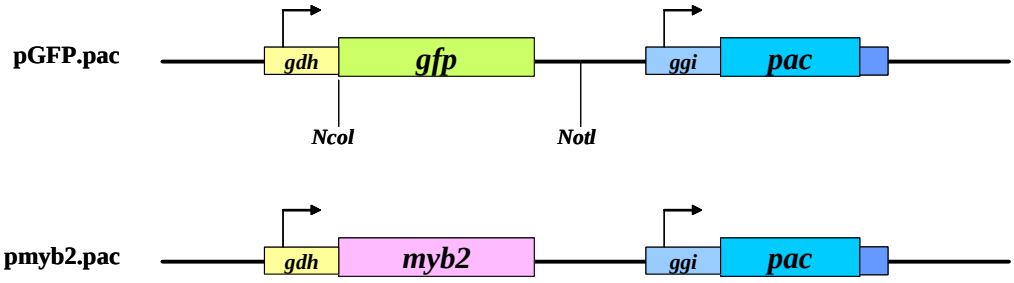


Fig. 1. Construction of a Myb2 overexpression plasmid of *G. lamblia*. The *myb2* gene was amplified from genomic DNA of *G. lamblia* WB was cloned into *Nco l/Not l* site of pGFP.pac.

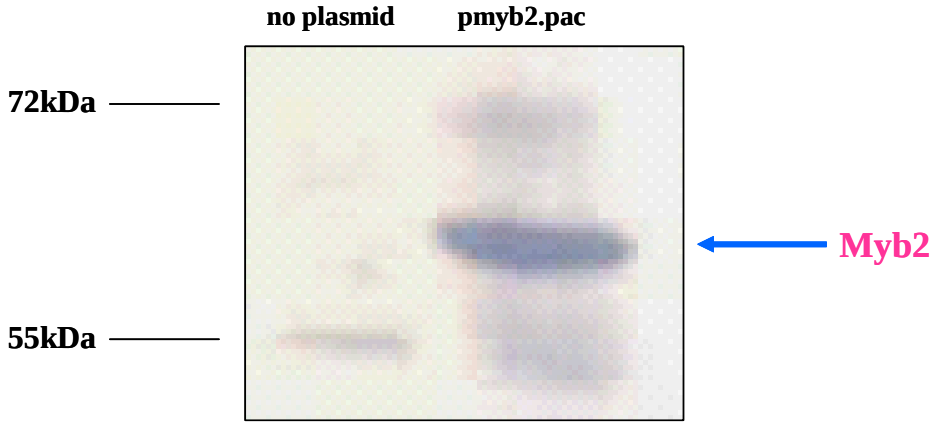


Fig. 2. Western blot analysis for Myb2 expression in *G. lamblia* carryng pmyb2.pac using polyclonal antibodies specific to the recombinant Myb2. In the extracts of *G. lamblia* with pmyb2.pac, Myb2 protein appears as an immunoreactive band. On the other hand, no immunoreactive band is present in the extract of *G. lamblia* trophozoites without any plasmid as expected.

2. Identification of up-regulated proteins by Myb2 overexpression

To find the up-regulated proteins by Myb2 overexpression, We was performed a two dimentional gel electrophoresis of the cell extracts of *G. lamblia* carrying pmyb2.pac in comparison with those of *G. lamblia* without any plasmid (Fig. 3). Cell extracts of *Giardia* with pmyb2.pac showed a dozen of increased protein spots in quantity when they were compared with the corresponding protein spots in two dimentional gel electrophoresis of the extracts of *G. lamblia* without plasmid. Ten protein spots showing increased intensity in *G. lamblia* carrying pmyb2.pac were isolated and analyzed by MALDI/TOF, and identified as listed in Table 3. With respect to their putative function, they were categorized as cytoskeletal proteins, metabolic enzymes, stress-resistance proteins, cell cycle related kinases, and hypothetical proteins.

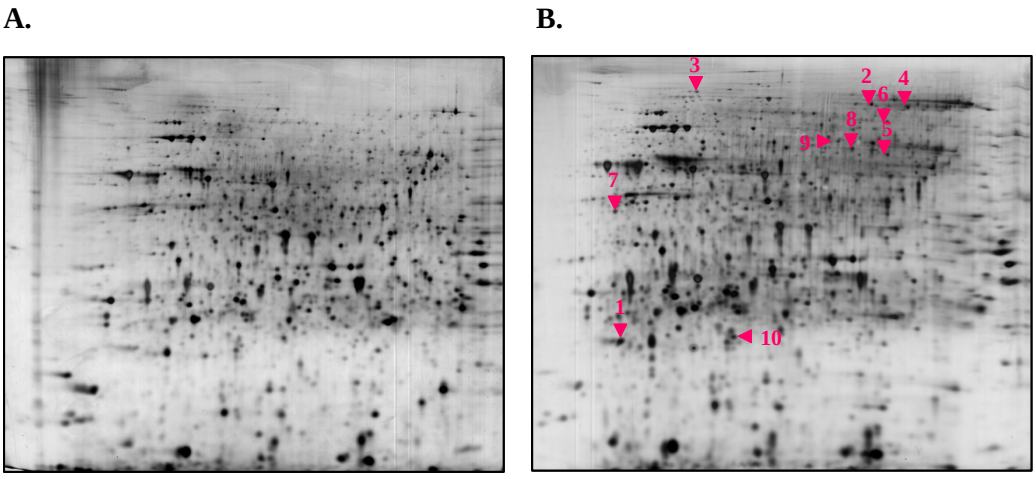


Fig. 3. Two dimentional gel electrophoresis of proteomes of *G. lamblia* trophozoites without any plasmid (A) and with pmyb2.pac (B). The arrows indicate the proteins up-regulated in *G. lamblia* carrying pmyb2.pac. Identities of these proteins were defined by MALDI/TOF analysis.

Table 3. Up-regulated proteins in *G. lamblia* carrying pmyb2.pac

protein name	spot #	MW (KDa)	score	E-value	fold change
tubulin-β chain	1	50.7	73	0.16	15
acyl-coA synthetase	2	100	135	$9.2e^{-8}$	6.2
ubiquitin-activating enzyme	3	123.1	113	$1.5e^{-0.5}$	4
phosphatedikinase	4	98.8	98	0.00042	3.7
chaperonin subunit α-CCT α *	5	59.7	107	$5.8e^{-0.5}$	3.4
G2-specific kinase fin 1*	6	77	146	$7.3e^{-0.9}$	3.34
arsenic resistance protein	7	39.8	113	$1.5e^{-0.9}$	3.32
myo-inositol 1-phosphatate synthase*	8	60.4	105	$9.2e^{-0.8}$	3
ORF 17060*	9	60.4	105	$9.2e^{-0.8}$	4.4
ORF 8329*	10	60.4	105	$9.2e^{-0.8}$	4.2

*indicates the proteins whose expression was monitored at the level of transcripton by real-time PCR.

3. Identification of up-regulated proteins in cyst.

The proteomic changes of *G. lamblia* during encystation were also examined by two dimentional gel electrophoresis (Fig. 4). Overall, a few dozens of protein spots varied in their amount, indicating that dramatic changes had been occurred in proteomic pattern during encystation of *G. lamblia*. Eighteen of the up-regulated proteins in encysting *Giardia* were identified, which belong to cytoskeletal components, stress-resistance proteins, metabolic enzymes, and a cell cycle related protein (Table 4).

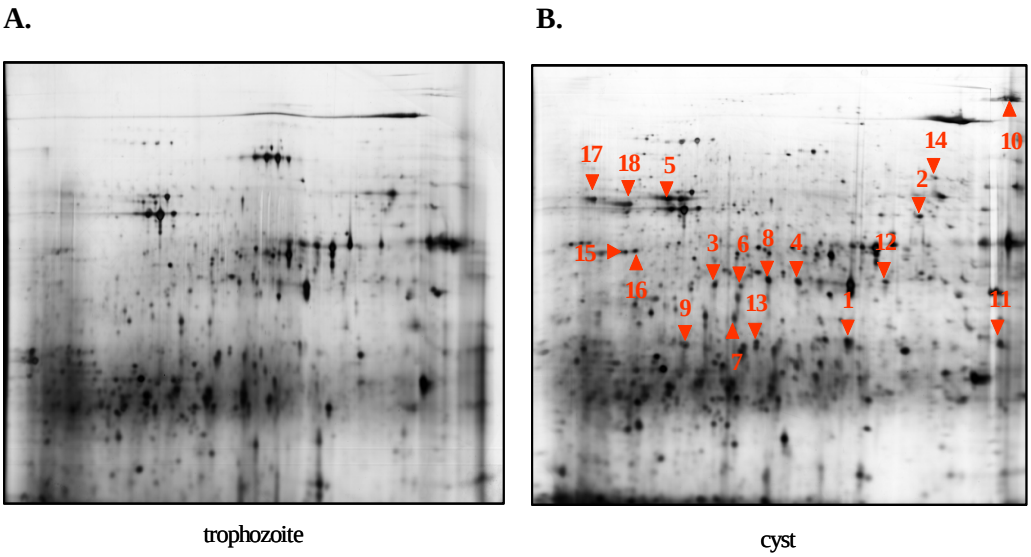


Fig. 4. Two dimentional gel electrophoresis of proteomes of trophozoite (A) and encysting cells (B) of *Giardia lamblia*. The arrows indicate the proteins up-regulated in encysting *G. lamblia*. Identities of these proteins are listed Table 4.

Table 4. Up-regulated proteins in *in vitro*-derived cyst

protein name	spot #	MW (KDa)	score	E-value	fold change
ornithine carbamoyltransferase	1	37.8	106	$3e^{-0.5}$	53
translation elongation factor, EF-1γ	2	45.8	112	$7.6e^{-0.6}$	46
α-tubulin	3	49.6	92	0.00072	45
	4	49.6	134	$4.8e^{-0.8}$	33
	5	49.6	111	$9.5e^{-6}$	13
	6	49.6	96	0.00031	11
	7	49.6	137	$2.4e^{-0.8}$	10
	8	49.6	120	$1.2e^{-0.6}$	5.4
giardin-β chain	9	30.9	107	$2.4e^{-0.5}$	45
pyruvate ferridoxin oxidoreductase	10	113.1	103	$6e^{-11}$	31
α-1-giardin	11	34.3	85	0.004	31
branched chain amino acid transferase	12	39.3	85	0.039	16
dipeptidyl aminopeptidase	13	83.3	95	0.00038	12

arginine deaminase	14	65	108	$1.9e^{-0.7}$	6
heat shock protein 90[#]	15	40.2	58	2.1	UD*
cytoplasmic 70kDa heat shock protein[#]	16	71.1	85	0.004	UD*
never in mitosis gene a-related kinase 1[#]	17	57.7	157	$2.4e^{-1.0}$	UD*
	18	57.7	121	$9.5e^{-0.7}$	UD*

*UD : unable to be detected

[#]indicates the proteins whose expression was monitored at the level of transcripton by real-time PCR.

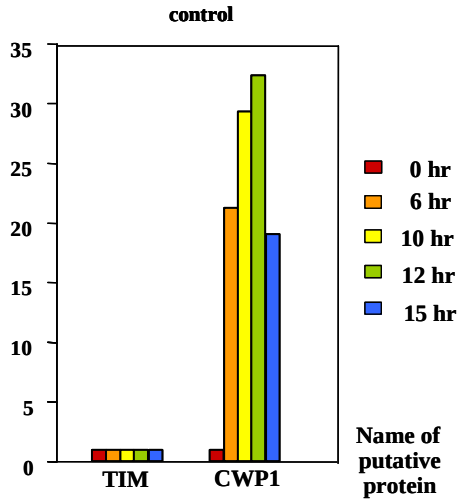
4. Quantitative analysis of transcripts of selected genes during encystation.

Eight were selected among the up-regulated proteins: five increased proteins in Myb2-overexpressing *Giardia* and three up-regulated proteins in encysting *Giardia*. We then examined whether the increased level of these proteins was caused from the increased expression of the corresponding genes. Using the specific primers designed to amplify these genes (Table 1), the transcript level of each gene was monitored by real-time PCR under trophozoite stages and various time-points of encystation (Fig. 5).

Expression of *cwp1* encoding cyst wall protein 1 was determined, and found to be increased 33-fold, confirming that induction of encystation occured properly under my experimental conditions. On the other hand, *tim* encoding triose 1-phosphate isomerase expressed in a rather constitutive mode. Therefore, all transcript levels were expressed as a normalized value to the amout of *tim* transcript of the identical RNA sample.

Expression of three genes encoding myo-inositol 1-phosphate synthase, chaperonin CCT-α, and G2-specific protein kinase fin1 was not increased upon induction to encystation. On the contrary, two genes for hypothetical proteins showed increased expression during encystation. With respect to three up-regulated proteins in the

encysting *Giardia*, two genes encoding heat shock proteins demonstrated increased expression during encystation. However, the gene for never in mitosis (NIMA) a-related kinase 1 expressed at a constant level, indicating that its isolation as an up-regulated protein in the encysting cells is an artifact of proteomic analysis.


control
35
30
25
20
15
10
5
0
TIM
CWP1
0 hr
6 hr
10 hr
12 hr
15 hr
Name of putative protein

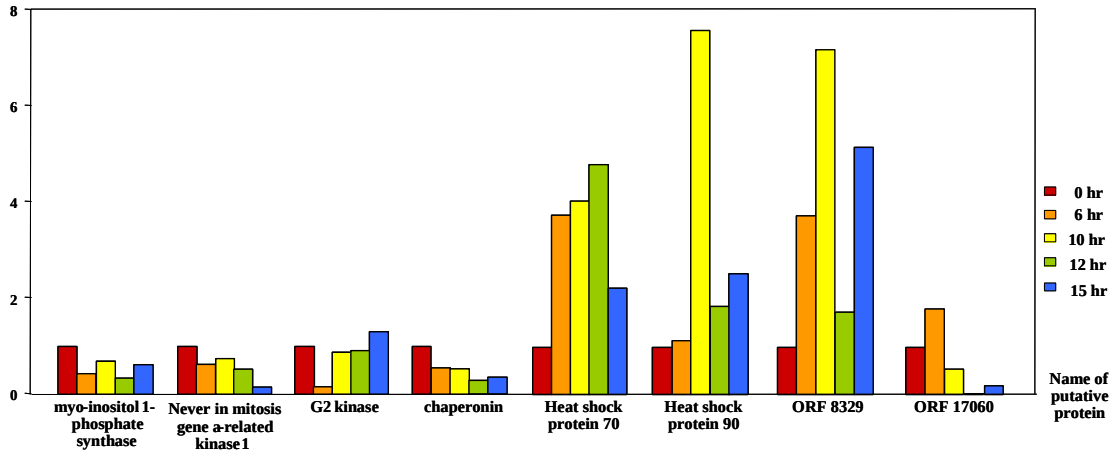

8
6
4
2
0
myo-inositol 1-phosphate synthase
Never in mitosis gene a-related kinase1
G2 kinase
chaperonin
Heat shock protein 70
Heat shock protein 90
ORF 8329
ORF 17060
0 hr
6 hr
10 hr
12 hr
15 hr
Name of putative protein

Fig. 5. Quantitative analysis of transcripts of selected genes during encystation. Expression of the *tim* gene was constitutive, whereas the expression of the *cwp1* gene was increased over 30-fold during encystation. Transcription of two heat shock proteins was induced during encystation. mRNA level of two transcripts with unknown functions was also increased during encystation.

5. Quantitative analysis of transcripts of selected genes in pmyb2.pac in trophozoites.

Role of Myb2 in transcription of the selected genes was determined by real-time PCR analysis of the Myb2-overexpressing *G. lamblia* (Fig. 6). In a transgenic *Giardia* carrying pmyb2.pac, the expression of the *myb2* gene was increased 18-fold, as expected. Transcript level of the *cwp1* was over 6-fold higher in the Myb2-overexpressing trophozoites compared to that in trophozoites without any plasmid. Expression of the *tim* gene was served as a loading control for RNA amount, and all transcript levels were expressed as a normalized value to the amout of *tim* transcript of the identical RNA sample.

Four of the 8 selected genes (encoding chaperonin CCT-α, G2-specific protein kinase fin1, heat shock protein 70, and a hypothetical protein ORF17060) showed increased expression by overexpression of Myb2. The rest four genes did not demonstrate any increase in their transcript level in the Myb2-overexpressing *Giardia*.

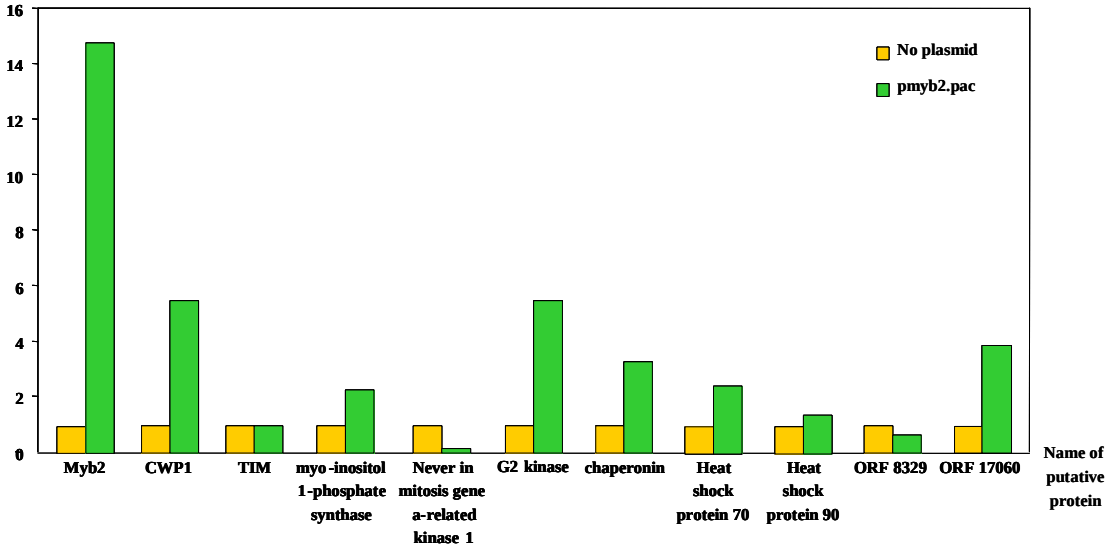


Fig. 6. Quantitative analysis of transcripts of selected genes in *G. lamblia* carrying pmyb2.pac. As expected, the expression of *myb2* and *cwp1* gene was increased in *Giardia* carrying pmyb2.pac. Transcription of heat shock protein 70 gene was slightly induced by overexpression of Myb2. A gene encoding a hypothetical protein ORF17060 showed increased expression in Myb2-overexpressing *Giardia*. Level of two transcripts encoding chaperonin CCT-α and G2 kinase fin1 was also elevated in *Giardia* carrying pmyb2.pac.

6. Gel-shift analysis of rMyb2 binding to promoter regions of Myb2-regulated genes.

In this experiment, We examined whether transcription of two possible Myb2-target genes (encoding G2 kinase fin1 and a hypothetical protein ORF17060) is mediated by direct interaction between Myb2 protein and their promoter region (Fig. 7). Binding of rMyb2 to the *cwp1* promoter indicated that experimental conditions for gel-shift assay were optimized. The same rMyb2 did not bind to promoter region of the *tim* gene, whose expression was known to be not affected by Myb2.

Promoter DNA of the *fin1* gene showed specific interaction with rMyb2. Their interaction was abolished with addition of competitor DNA, the unlabeled *fin1* promoter, but maintained intact with excess amount of *tim* promoter DNA. In the same manner, the promoter of a ORF17060 demonstrated rMyb2 binding, which seemed to be specific based on the competition experiment.

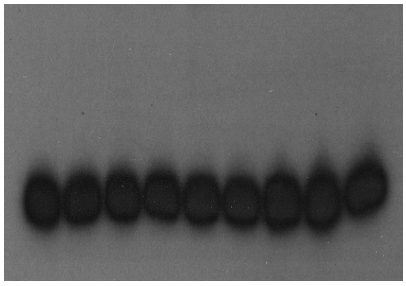
A.

cDNA : - - - - - ▲ +

rMyb2 : - - + + + + + + +

BSA : - + - - - - - - -

DNA : ***tim*** **promoter**



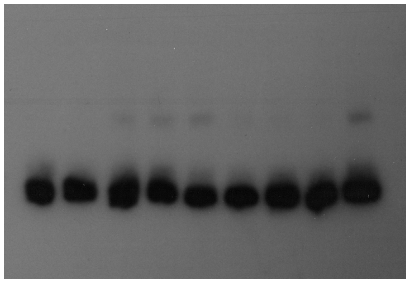
B.

cDNA : - - - - - ▲ +

rMyb2 : - - + + + + + + +

BSA : - + - - - - - - -

DNA : ***cwp1*** **promoter**



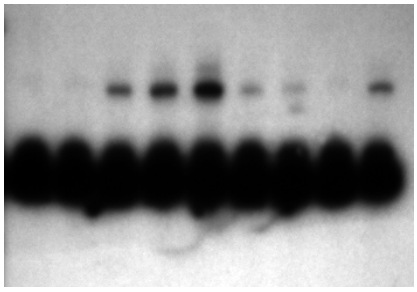
C.

cDNA : - - - - - ▲ +

rMyb2 : - - + + + + + + +

BSA : - + - - - - - - -

DNA : ***17060*** **promoter**



D.

cDNA : - - - - - ▲ +

rMyb2 : - - + + + + + + +

BSA : - + - - - - - - -

DNA : ***fin 1*** **promoter**

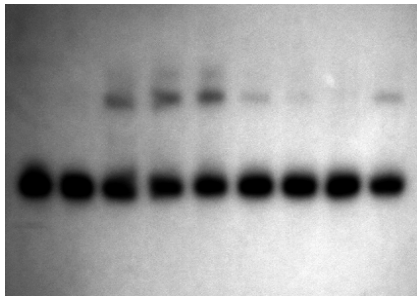


Fig. 7. Gel-shift analysis of rMyb2 binding to Myb2-target promoters. (A-D) ^{32}P-labeled *tim, cwp1,* ORF17060, and *fin1* promoters were incubated without rMyb2 (lane 1), with 1 μg BSA (lane 2), or 50, 80, 100 nM rMyb2 (lane 3-5). For lanes 6-8, the ^{32}P-labeled *tim, cwp1,* ORF17060, and *fin1* promoters were reacted with 100 nM rMyb2 in the presence of increasing amounts of unlabeled *tim* as a competitor.

Ⅳ. DISCUSSION

The most important clinical symptoms of giardiasis are diarrhea and malabsorption. Despite the pathophysiology associated with these symptoms is still incompletely understood [48-49], intestinal colonisation by this parasite seems to cause microvillus shortening [50-52], villous flattening or atrophy [53]. These histological abnormalities, possibly in combination with other pathological mechanisms such as reduction of intestinal disaccharidase [54] and protease activities [55], may be direct causes of clinical symptoms in giardiasis [56].

Because *Giardia* colonizes the intestinal tract, it must constantly react to changing signals from the host environment. Once trophozoites are carried to the end of the small intestine, they rapidly differentiate into cysts to survive outside of the host (encystation). Despite its importance in modulating giardial responses to its changing environments, regulation of gene expression during this process is poorly understood. Distinct formation of extracellular cyst wall and encystation-specific vesicles is the key process in encystation, however, little is known how they are regulated [29, 57].

Since *G. lamblia* is the earliest branching point of eukaryotes, it emers as an ideal model organism to study the early forms of eukaryotes [12, 21]. Comparison of

transcriptional machinery in Archaea and Eukarya suggests that both lineages contains a core transcription apparatus composed of a single RNA polymerase and a recruitment mechanism corresponding to a TATA-binding protein (TBP)/transcription factor IIB (TFIIB)-type complex. The single archaeal RNA polymerase shares a specific relationship with the three eukaryal polymerases, exemplified by shared subunit composition and a common architecture [41]. Basal initiation in archaeal transcription is dependent upon two factors, TBP and TFB (the archaeal homolog of TFIIB), recognizing TATA elements of gene promoters [58]. However, the eukaryal transcription apparatus has undergone extensive elaboration, unparalleled in the evolution of archaeal transcription. Eukaryotes use three different RNA polymerases (RNAPI, RNAPII, and RNAPIII) to transcribe different classes of genes, generally ribosomal RNAs, messenger RNAs, and transfer RNAs, respectively; each RNAP initiates transcription at specific gene promoters aided by different sets of transcription factors. A unique opportunity to examine the components of the transcription apparatus in a potentially early diverging eukaryote stems from the genome-sequencing project of the human parasite *Giardia lamblia*. Phylogenetic analyses of small subunit rRNA [2], translation elongation factor (EF) 1α [3], EF-2 [4], and eukaryotic release factor 3 support an early divergence of *Giardia* from

the eukaryotic line of descendent, although the basal position of *Giardia* in some phylogenetic reconstructions has been questioned. In addition, certain morphological features of *Giardia* cells hint at altered or simpler forms of characteristics found in recently diverging organisms. Regardless, it is most likely that divergent features of the transcription machinery observed in *Giardia* trace back to very early stages of eukaryal evolution [21].

Little is known about transcription mechanism of *G. lamblia*. Previous studies suggest that Myb2 is an encystation-induced transcription factor, raising a possibility that it may play an essential role in encystation. Therefore, we searched target genes controlled by Myb2 using comparative proteomic analysis. Eight genes were chosen to examine their expression during encystation and dependence of their expression on Myb2 (Table 5).

Table 5. Grouping of the isolated proteins according the expression pattern

Protein name	Encystation-induced	Myb2-induced
Never in mitosis gene a-related kinase 1	No	No
Chaperonin subunit α-CCT α	No	Yes
Myo-inositol 1-phosphatate synthase	No	No
Heat shock 70	Yes	Yes
Heat shock 90	Yes	No
G2-specific kinase fin1	No	Yes
ORF8329	Yes	No
ORF17060	Yes	Yes

Two of them, genes for NIMA and myo-inositol 1-phosphate synthase, did not show any increased expression during encystation and in Myb2-overexpressing *G. lamblia*, indicating that they are taken out as an artifact of proteomic analysis. The genes encoding heat shock 70 and ORF17060 were found to be controlled by both encystation and Myb2. Two genes encoding heat shock 90 and ORF8329 showed increased expression during encystation, however, their expression is not induced by

Myb2. Thus, they raise a possibility for involvement of other transcriptional factors in their expression. Interestingly, genes for CCT-α and G2-specific kinase fin1 expresses in Myb2-overexpressing cells whereas their expression is not induced by encystation. This result suggests that Myb2 may function in other process besides encystation.

We also examined binding ability rMyb2 to the promoters of the encystations-induced genes (Fig 7). While the promoter of the *tim* gene, constitutively expressed in trophozoites and encysting cells, did not bind to rMyb2 at all, promoter of the *cwp1* and two genes (for ORF17060 and G2 kinase) were bound with rMyb2. Identification of basal transcriptional machinery of *G. lamblia* is pre-requisite to improve the affinity and specificity of rMyb2 binding to these promoters.

In this study, the role of Myb2 in encystation was examined by finding its target genes. I extend the list of Myb2-target genes by adding two genes encoding heat shock 70 and ORF17060 in *G. lamblia*.

Ⅴ. CONCLUSIONS

1. *G. lamblia* carrying pmyb2.pac shows increased expression of Myb2 by western blot analysis using polyclonal antibody specific to the Myb2.

2. A group of proteins encoding cytoskeleton proteins, metabolic enzymes, cell cycle related kinases, stress-resistance proteins, and hypothetical proteins show up-regulation in proteomes of cyst and Myb2-overexpressing *G. lamblia*.

3. Real-time PCR measurement of transcript levels demonstrates that genes encoding heat shock protein 70 and ORF17060 demonstrate induced expression during encystation as well as in Myb2-overexpressing *G. lamblia*.

4. Gel shift assays indicate that rMyb2 directly binds to the promoter region of gene for ORF17060.

5. The list of Myb2-target genes is extended by adding two genes encoding heat shock protein 70 and ORF17060.

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ABSTRACT (IN KOREAN)

람블편모충 단백질체 비교분석을 통한 전사인자 Myb2의 기능에 대한 연구

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방 소 현

인간에게 설사병을 일으키는 람블편모충은 영양형과 포낭으로 이루어진 생활사를 가지고 있다. 영양형에서 포낭으로 전환되는 피낭유도과정에 관한 이전의 연구에서 전사인자로 추정되는 *myb*2는 피낭유도과정 유도 유전자라고 밝혀졌다. Myb2 과발현 transgenic 람블편모충을 이용하여 Myb2 단백질에 의해 조절 받는 표적 유전자를 찾았다. Myb2 과발현 plasmid인 pmyb2.pac은 pGFP.pac의 *gfp* 유전자를 *myb2* 유전자로 교체하여 제조되었다. 2차원 젤 전기영동을 통해, pmyb2.pac을 수반하는 람블편모충 영양형의 단

백질체와 어떠한 plasmid도 넣지 않은 람블편모충 영양형의 단백질체를 비교하였다. 또한, 영양형과 피낭유도과정 중의 람블편모충의 단백질체도 비교하였다. pmyb2.pac을 수반하는 람블편모충에서는 10개의 단백질의 발현이 증가되었고, 피낭유도과정 중의 람블편모충에서는 18개의 단백질이 증가되었는데 이런 증가된 단백질은 세포골격, 물질대사 효소, 세포주기 특이 kinase, 스트레스 저항 단백질과 두 개의 hypothetical open reading frame으로 분류되었다. 이 중, 8개의 유전자를 선택하여 real-time PCR을 통해 피낭유도과정과 Myb2 과발현 transgenic 람블편모충에서의 발현을 알아 보았다.

cwp1 (encoding cyst wall protein 1) 유전자의 발현은 피낭유도과정의 positive control로 사용한 반면, *tim* (triose-1-phosphate isomerase) 유전자의 발현은 RNA 양에 대한 loading control로 사용하였다. *cwp1* 유전자의 발현은 피낭유도과정 동안에 33배 이상 증가하였지만, *tim* 유전자는 항상 일정량이 발현되는 양상을 보였다. 열충격단백질 70과 열충격단백질 90을 발현하는 두 스트레스 반응 유전자와 두 hypothetical 유전자는 피낭유도과정에서 2배에서 7배 정도 증가된 것으로 보였다. 과발현 Myb2 람블편모충에서는 오직 열충격단백질 70과 hypothetical protein (ORF17060)이 3배에서 4배정도 증가되었다. Gel-shift assay로 *cwp1* 유전자, ORF17060, G2-specific kinase fin1의 promoter 지역에 재조합 Myb2 단백질이 결합된다는 것을 밝혔다. 이 결과

로 열충격단백질 70과 ORF17060의 유전자는 Myb2 단백질에 의해 조절되는 표적유전자라는 것을 제안한다.

핵심이 되는 말 : 람블편모충, Myb2, 피낭유도과정